THE METABOLISM OF PROGESTERONE BY ANIMAL TISSUES IN VITRO

A Thesis presented for the Degree

of

DOCTOR OF PHILOSOPHY

by

William Taylor, B.Sc.

Department of Biochemistry

University of Edinburgh.

1953.

ACKNOWLEDGEMENTS

The author wishes to record his appreciation of the advice and encouragement of Professor G.F. Marrian, F.R.S. and Dr. J.K. Grant under whose supervision these investigations were carried out.

He is also indebted to Dr. W.S. Bauld for instruction in the principles and practice of partition chromatography and counter-current distribution.

Thanks are also due to Dr. J.W. Minnis who carried out the micro-analyses and to Mrs. S.M. Atherden who performed the formaldehyde estimations.

CONTENTS

Page.

SECTION II

GENERAL INTRODUCTION

It is not the author's intention to attempt a comprehensive survey of the biochemistry of progesterone as the subject has been fully treated in a number of reviews of recent date (Pearlman, 1948; Marrian, 1949; Bradbury, Brown and Gray, 1950 and Samuels and West, 1952). It will suffice, therefore, to summarise the salient points concerning the fate of progesterone in the animal body and to deal more fully with those aspects of the subject with which the present investigations are concerned.

Until recently, the main approach to the study of progesterone metabolism has been by the examination of urine, bile and faeces for compounds that resemble the hormone chemically. Investigations of this type are handicapped by the low concentration and complexity of mixtures of the steroid metabolites encountered and also by the lack of sufficiently accurate and sensitive methods for the quantitative determination of such compounds. These difficulties have to some extent been overcome by:

i) increasing the amounts of metabolites

Table I

Urinary Steroids related to Progesterone

A.

Pregnane-3:20-dione
5α-Pregnane-3:20-dione

Pregnan-3α-ol-20-one
5α-Pregnan-3α-ol-20-one
5α-Pregnan-3β-ol-20-one

Pregnane-3α:20α-diol
Pregnane-3β:20α-diol
5α-Pregnane-3α:20α-diol
5α-Pregnane-3β:20-diol

Pregnan-3α-ol

B.
Pregn-5-ene-3β:20α-diol
5α-Pregnane-3β:16α:20β-triol
5α-Pregnane-3α:6α-diol-20-one
Pregnane-3α:6α-diol-20-one
17α-Pregnan-3α-ol-20-one

(Throughout this Thesis, steroid nomenclature follows the rules proposed by a conference at the CIBA Foundation, London, and published in J. Chemical Society (1951), p. 3526).

excreted by administering relatively massive doses of progesterone,

ii) by studying the steroid content of pregnancy urines,

iii) by the quantitative determination of urinary pregnane-3α:20α-diol (pregnanediol), a steroid which is amenable to analysis owing to its possessing distinctive chemical and physical properties.

Since only ten to fifteen per cent of exogenous progesterone is recovered as urinary pregnanediol, the usefulness of such determinations in studies on progesterone metabolism, must be open to question.

In vivo metabolism of progesterone

Table 1 lists the steroids which have been isolated from human pregnancy urines (see Samuels and West, 1952, for references) and which are assumed to be metabolites of progesterone. Of these, however, only three have been shown conclusively to be derived from progesterone viz. pregnanediol and pregnan-3α-ol-20-one (pregnanolone), (Dorfman, Ross and Shipley, 1948) and 5α-pregnane-3α,20α-diol (Kyle and Marrian, 1951).

Pregnanolone and pregnane-3α:20β-diol have

been isolated from cow bile following progesterone administration and pregnanediol was found in the bile of a post-menopausal woman after oral administration of pregn-5-en-3β-ol-20-one (pregnenolone), a hypothetical precursor of progesterone (Pearlman and Cerceo, 1948; Pearlman and Pincus; 1946). Pregnane-3β:20α-diol has been isolated from human bile after oral administration of progesterone (Rogers and McLellan, 1951).

Only pregnanediol has been isolated from faeces. Hoffman and Browne (1942) recovered this steroid from the faeces of rabbits following oral but not subcutaneous administration, of progesterone. That the faeces may be an important route for the excretion of progesterone metabolites in some animals has been shown by the results of Riegel, Hartop and Kittinger (1950) and Grady, Elliot, Doisy, Bocklage and Doisy (1952). When ^{14}C-21-progesterone was administered to rats and mice, most of the radioactivity was recovered from the faeces.

It is customary to arrange the steroids in Table 1(A) in a scheme which suggests that these compounds are produced by stepwise reduction of progesterone by a more or less regular process. The arrangement is based on the close resemblance between the metabolites and the hormone and also on the

simple chemical reactions which would be necessary to convert one to the other. Such schemes, however, cannot be firmly established by isolation studies alone. The compounds listed in Table 1 might arise from adrenal cortical steroids by, for example, reduction at C-11 and C-21. It is well established that administration of 11-deoxycorticosterone (DOC) leads to increased excretion of pregnanediol in a number of species, including man. (See Schneider and Horstmann, 1951, for references). Furthermore, progesterone itself might be converted into metabolites possessing an oxygen function at C-21 or by removal of the side chain, into C_{19} steroids.

In vitro metabolism of progesterone

In vivo studies have indicated that the sex organs and adrenals are not the major sites of conversion of progesterone to pregnanediol and that the liver plays an important role in the inactivation of the hormone (Samuels et al., 1952). The results of early in vitro experiments, however, appeared to indicate that liver preparations are unable to metabolise progesterone (Zondek, 1941: Engel, 1944). Using more sensitive chemical techniques, Samuels, McCaulay and Sellers (1947) were able to show that

the α-β-unsaturated ketonic group of progesterone underwent some change on incubation with rat liver mince.

In vitro studies with adrenal preparations have indicated that progesterone may be a key intermediate in the biosynthesis of adrenal cortical hormones. Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker and Pincus (1951) perfused progesterone through isolated beef adrenals. Of the perfused progesterone, 53 per cent was recovered as corticoids and of this, 20 per cent was unidentified, 17 per cent was 17-hydroxy-corticosterone and 16 per cent corticosterone. Hayano and Dorfman (1952b) have demonstrated the conversion of progesterone to corticosterone by ox adrenal homogenates. Plager and Samuels (1952: 1953) found that the supernatant obtained after centrifugation of ox adrenal homogenate at 20,000 x g for 30 min., could convert progesterone to DOC and 17-hydroxyprogesterone. The mitochondrial fraction contained an enzyme capable of introducing an hydroxyl group at C-11.

It is apparent then, that progesterone may undergo many varied metabolic reactions in different organs. In vivo studies are open to a number of limitations since there is always the question of the extent to which endogenous metabolism is affected

by the administration of exogenous steroid. The problem as to the number of organs involved can partially be simplified by removal of one or more of these organs but more cannot be removed without the possibility arising of secondary changes influencing the result. Furthermore, the routes of excretion may differ in different species and the selective character of the kidneys is introduced in all. In vivo studies can provide a great deal of useful information in spite of these limitations but for our understanding of the biochemical significance of steroid hormones to be extended, such studies require to be supplemented by in vitro experiments.

The purpose of the present investigation was to ascertain the site or sites of conversion of progesterone to pregnanediol. A series of exploratory experiments directed to this end yielded negative results and so a method was developed for the determination of progesterone in the presence of incubated tissue preparations. This method appears to have many advantages over the techniques employed by other workers in this field of study.

A preliminary study of the metabolism of progesterone by rabbit and rat liver has indicated that diphosphopyridine nucleotide (DPN) plays an

important role.

A beginning has been made on the isolation of progesterone metabolites. Two metabolites, 5α-pregnane-3:20-dione and 5α-pregnan-3α-ol-20-one have been isolated and indications are that at least two more metabolites are formed.

While the present investigations were in progress, Wiswell and Samuels (1953) published the results of their work on *in vitro* metabolism of progesterone by rat liver. They have not reported the isolation of metabolites and their results are at variance with the author's in that they report that DPN is not involved in the metabolism of progesterone *in vitro*.

GENERAL METHODS AND MATERIALS

a) Experimental animals

Rats were females of the Wistar strain, 6 - 12 months old. They were killed by dislocation of vertebrae in the neck and the livers, rapidly dissected out, were placed in a small beaker containing ice-cold, isotonic potassium chloride solution. When the liver was to be homogenised in potassium chloride-nicotinamide solution then the same solution was used for the initial chilling of the liver.

Rabbits used were of a variety of strain, age and sex. Unless otherwise stated they were killed by stunning and exsanguination.

Incubations were normally begun within five minutes of the death of the animal.

b) Liver preparations

i) Slices

Slices of liver about 0.3 mm. thick were prepared by the method of Deutsch (1936) as modified by Cohen (1945). They were not immersed in any solution but were placed when cut in a covered Petri dish standing on ice. A moist atmosphere was maintained in the dish by lining the bottom with hardened

filter-paper moistened with 'phosphate saline'. The filter-paper was marked off in sectors corresponding to the number of samples required, and in order to ensure uniform sampling, slices were piled serially in the sectors. Samples were weighed on a torsion balance to the nearest 2.0 mg.

ii) Homogenates

After being chopped roughly with scissors, the required amount of tissue was weighed into the mortar of the homogeniser standing in crushed ice and 6 ml. of ice-cold, 0.15M-potassium chloride (unless otherwise stated) were added. At the beginning of these investigations, all-glass homogenisers (Potter and Elvehjem, 1936) were used but later, pestles manufactured locally from nylon rod were employed. Brendler (1951) constructed a homogeniser consisting of a glass homogenising chamber and a "lucite" pestle. He found that the homogenates prepared in this apparatus were more uniform than those prepared in all-glass types. An added advantage is that there is no apparent change in the clearance between the shearing surfaces after a period of many months, whereas the ground surfaces of all-glass homogenisers wear rapidly.

When all-glass homogenisers were used, the

tissue was ground for one minute with four passages of the pestle; six passages of the nylon pestle were found necessary for uniform homogenisation. The final homogenate was diluted to 10 ml. with ice-cold potassium chloride solution (with or without nicotinamide depending on the experiment) and was kept standing in ice water until pipetted into the incubation flasks. When more than 10 ml. of homogenate was required, the desired volume was obtained by bulking 10 ml. batches prepared as described.

c) Suspension media

All solutions used in enzymic experiments were prepared in glass-distilled water and all reagents were of A.R. grade. The suspension medium used throughout and referred to as 'phosphate saline' was the calcium-free phosphate saline (pH 7.4) of Krebs and Eggleton (1940)

d) Diphosphopyridine nucleotide (DPN)

DPN was prepared by the method of Le Page (1949) and its purity determined periodically by spectrophotometry (Le Page, 1947). Stored in a

stoppered bottle over P_2O_5 at -20°, the preparation retained a purity of 37 - 39% for over two years. Concentrations of DPN in reaction mixtures were calculated from the actual weight added.

e) Measurement of pH

All measurements of pH were made on a battery-operated pH meter (Type D-417. Muirhead & Co. Ltd).

f) Addition of "co-factors"

Unless otherwise stated, these were added in 'phosphate-saline' solution, after adjustment of pH to 7.4 if necessary. The final volume of the reaction mixture was maintained by reducing the volume of "pure" 'phosphate saline' in the flasks.

g) Addition of steroid

The most commonly used method for the addition of "free" steroids to incubation mixtures has been to dissolve the steroid in a minimum amount

of organic solvent and to add the solution to incubation flasks by means of a micropipette. Samuels et al. (1947) employed ethanol solutions in their studies on testosterone metabolism. Schneider et al. (1951) found that 11-deoxycorticosterone forms relatively stable supersaturated solutions suitable for pipetting, when an ethanolic solution of the steroid is added to hot saline. The use of ethanol might be objected to in view of the findings of Kochakian (1951) that ethanol, in low concentration stimulates the respiration of liver and kidney slices while at higher concentration, respiration is inhibited. Propylene glycol (1:2-dihydroxypropane) is being used increasingly in place of ethanol (Seneca et al., 1950; Kahnt and Wettstein, 1951; Wiswell et al., 1953). This alcohol is converted to lactate and to propylene glycol glucuronide in vivo (Williams, 1949) and it is not unlikely that it undergoes some change in vitro. Its high solvent power for steroids, however, permits the addition of only very small volumes and for this reason, it may be regarded as a suitable solvent for in vitro work. Therefore, except in the experiments described in Section I, progesterone was added to incubation flasks as a solution in propylene glycol.

h) The general arrangement of incubation experiments

All incubation, control and blank vessels were prepared in duplicate. Except where otherwise stated the following procedures were adopted.

Incubations: The required volume of chilled 'phosphate saline' (3.0 ml. for slices, 2.0 ml. for homogenate experiments) was pipetted into 25 ml. wide-necked conical flasks standing on ice. Slices or homogenate (1.0 ml.) were added followed by the steroid solution. When all the flasks had been prepared, they were gassed for 30 sec. when necessary, closed with rubber stoppers and transferred to a water bath at 37°. Shaking at a frequency of 90 - 100/min. was continued throughout the incubation period.

Steroid recovery controls ("Controls"): Incubated tissue was added to the steroid suspension in 1.0 ml. of 'phosphate saline' which had been incubated with co-factors (when used) and the working up procedure begun at once. "No-steroid" blanks ("Blanks") were treated in the same way as incubations, with the exception that only the steroid dispersing solvent was added.

i) Steroids

i) Progesterone was the commercial product of Organon Laboratories. It was recrystallised twice from n-hexane and twice from aqueous ethanol. M.p. 121-121.5°.

Calculated for $C_{21}H_{30}O_2$:	C,	80.2,	H,	9.6
Found:	C,	80.2,	H,	9.3
		80.0,		9.1

ii) Pregnan-3α-ol-20-one was prepared from the ketonic fraction of sodium "pregnanediol" glucuronidate by the methods of Marrian and Gough (1946) and Sutherland and Marrian (1947). The product after two recrystallisations from hexane gave fine, white needles, m.p. 148 - 149°. Mixed with authentic pregnanolone (m.p. 146 - 148°), the m.p. was 146 - 147°.

iii) Pregnane-3α:20α-diol (Parke-Davis) was recrystallised from aqueous ethanol. M.p. 236.5 - 238°.

iv) 5α-Pregnane-3:20-dione was prepared by reduction of pregn-5-en-3β-ol-20-one (British Schering) with hydrogen and a platinum oxide catalyst, followed by oxidation with chromic oxide (Marker, Kamm and McGrew, 1937)

v) Testosterone (Organon Laboratories) was recrystallised from light petroleum (40 - 60°) and had m.p. 153 - 154.5°.

j) Melting points

All melting points were determined on an improvised hot-stage apparatus of the Kofler type (Klyne and Rankeillor, 1947). The apparatus was calibrated with pure substances of known melting point. The melting points recorded are therefore corrected.

k) Specific rotations

These were determined for the D line using a 0.5 dm. microtube. The errors are calculated as described by Klyne and Patterson (1948) except that fifteen pairs of readings with solution and fifteen pairs with a solvent blank were taken in each case.

SECTION I

ATTEMPTS TO DEMONSTRATE THE CONVERSION OF PROGESTERONE AND PREGNANOLONE TO PREGNANEDIOL

i) Introduction

As already stated in the General Introduction, it is well established that pregnanediol and pregnanolone are metabolic products of progesterone in many species, though the site of metabolism remains obscure. Grant and Marrian (1950) have described a method for the determination of "pregnanediol"* in the presence of incubated liver preparations and it seemed possible that the conversion of progesterone to pregnanediol in vitro might be demonstrated by means of this method. At the time that the present investigation was begun, nothing was known concerning the optimum conditions, co-factors, etc. necessary for the conversion to occur and so the exploratory experiments to be described were performed. This part of the work was carried out in conjunction with other aspects of pregnanediol metabolism (not reported herein) and does not represent a serious approach to the problems

* The term pregnanediol" is used to denote the material consisting largely but not necessarily entirely of pregnane-3α:20α-diol as determined by the method of Sommerville, Gough and Marrian (1948).

of progesterone metabolism *in vitro*.

ii) Incubation of aqueous suspensions of progesterone and pregnanolone with tissue preparations

In view of the small quantities of "pregnanediol" likely to be encountered, the method of Grant *et al.* (1950) was slightly modified. The whole of the alcohol filtrate obtained after precipitation and charcoal treatment was evaporated to dryness and the residue treated with concentrated H_2SO_4.

a) Incubation of progesterone with rabbit uterus

In small test-tubes, 2.5 mg. portions of progesterone were shaken for 2 hr. with a speck of crude ox bile (to facilitate dispersion) and 2.5 ml. 'phosphate saline'. A portion of the horn of the uterus was removed from a pregnant rabbit immediately after death which occurred while the animal was under nembutal anaesthesia. The specimen (4.0 g.) was minced finely with scissors and homogenisation in water attempted. The tissue was found to be too tough to disperse effectively and the final homogenate contained much whole tissue. Each reaction

vessel contained 1.0 ml. uterus "homogenate" (equivalent to 400 mg. tissue) and 1.5 ml. 'phosphate saline' suspension of progesterone (containing approximately 1.5 mg. steroid) to which had been added 5 mg. DPN and 35 mg. nicotinamide per 1.5 ml. Incubations were in nitrogen for 2 hr.

Results

"Pregnanediol" formed (µg.)

After incubation	In "controls"	In "blanks"
25	5	5
10	5	5

b) Incubation of progesterone with rat liver

Schneider and Horstman (1951) and Schneider (1952) have studied the metabolism of 11-deoxycorticosterone (DOC) by rat liver *in vitro* and have demonstrated the formation of four 5 - α pregnane derivatives. This conversion took place in oxygen and was partially inhibited in nitrogen. In order to disperse the steroid, Schneider added an ethanolic solution to hot saline and found that the supersaturated solution so obtained was quite stable. Because of the close similarity between the structures of progesterone and DOC, an experiment under

similar conditions was carried out.

Progesterone (15 mg.) was dissolved in 0.25 ml. hot ethanol and 25 ml. hot 'phosphate saline' were added, whereupon the steroid precipitated out in large floccules. The mixture was shaken vigorously to disperse the progesterone and 2.5 ml. volumes were pipetted into the incubation flasks standing in the bath at 37°. After a few moments had elapsed to allow the suspension to cool down to 37°, homogenate was added. Each reaction vessel contained 1.0 ml. homogenate (equivalent to 350 mg. liver), 2.5 ml. steroid suspension and in certain cases, 5 mg. DPN and 35 mg. nicotinamide or 5 mg. glutathione. Incubations were in oxygen for 2 hr.

Results

	"Pregnanediol" formed (µg.)		
	After incubation	In "controls"	In "blanks"
No added co-factors	16 11		
With added DPN and nicotinamide	15 18	12 12	10 10
With added glutathione	9 11	11 11	10 10

c) Incubation of pregnanolone with rat liver

As it seems probable that pregnanediol is formed by reduction of pregnanolone at the C-20 ketone group it was decided to incubate pregnanolone with rat liver homogenate in an attempt to demonstrate this reduction *in vitro*.

Pregnanolone (10.5 mg.) was dissolved in 0.25 ml. hot ethanol and 17.5 ml. of almost boiling 'phosphate saline' were added. The flocculent precipitate was dispersed by vigorous shaking and the suspension added to the reaction vessels in the manner described for the previous experiment. Each reaction vessel contained 2.5 ml. steroid suspension (containing 1.5 mg. pregnanolone) and 1.0 ml. rat liver homogenate (equivalent to 350 mg. tissue). One pair of flasks contained 5 mg. DPN and 35 mg. nicotinamide and was gassed with oxygen and the other pair contained 5 mg. glutathione and was gassed with nitrogen. Incubations were for 2 hr.

"Pregnanediol" formed (µg.)

	After incubation	In controls
With added DPN and nicotinamide	9 9	10 9
With added glutathione	9 9	9 9

iii) Discussion

In the experiments in which progesterone was incubated with rabbit uterus and rat liver there was apparently a very slight formation of "pregnanediol". It seemed rather surprising that no "pregnanediol" could be detected after incubation of pregnanolone in view of the relationship of this steroid to progesterone and pregnanediol.

Because of the small amount of "pregnanediol" formed it was felt to be unwise to draw any definite conclusions from these experiments. It seemed possible that the low conversion of progesterone might be a result of the low solubility of the steroid in the aqueous medium preventing it from being available to the enzyme(s) involved. (Bischoff and Pilhorn, 1948, report the solubility of progesterone in water after 140 hr. equilibration to be 11.4 µg./ml.) Attempts were therefore made to develop a medium able to solubilise progesterone and suitable for in vitro studies.

iv) Methods used for the dispersion of steroids and their possible application to in vitro studies with progesterone

a) Water-soluble derivatives

In order to eliminate the necessity for using a dispersion medium, many workers have favoured the use of water-soluble derivatives of steroids. Hemisuccinates have been the most widely used derivatives of alcoholic steroids, e.g. androst-5-en-3β-ol-17-one, androsterone and testan-3α-ol-17-one (Schneider and Mason, 1948a, 1948b), oestradiol (Pearlman and De Meio, 1949), pregnanediol (Grant et al., 1950) and androstane-3α:17β-diol (Kochakian and Aposhian, 1952). Schneider et al. (1948a) have shown that the rate of hydrolysis of this ester by liver esterases is rapid and not a limiting factor.

Glucuronic acid conjugates of steroid alcohols provide physiological derivatives which are water-soluble and Grant (1950) has shown that pregnanediol in the form of sodium pregnanediol glucuronidate (NaPG) is metabolised by liver in vitro. Glucuronides, however, are difficult and tedious to obtain; NaPG is especially so because of contamination with sodium pregnanolone glucuronidate

(Sutherland and Marrian, 1947).

Hayano, Dorfman and Prins (1949) demonstrated that 11-deoxycorticosterone glucoside is more readily converted to glycogenic material by adrenal homogenates than is the free steroid, presumably because of the greater availability of the steroid to the enzyme as a result of the higher solubility in water of the glucoside.

Progesterone presents the serious disadvantage that no easily-prepared, water-soluble derivatives of a physiological nature are available. Lieberman (1946) has shown that some 3-ketosteroids form thiazolidines with cysteine but he was not able to prepare the thiazolidine of progesterone. Lieberman and Dobriner (1948) have suggested that ketosteroids may be excreted in urine conjugated with cysteine but as yet, this has not been supported by the detection of such conjugates in urine. That 3-ketosteroids also form condensation products at the 3-position with thioglycollic acid has been shown by Jones, Webb and Smith (1949). Though such derivatives might be expected to be more water soluble than the free steroids, it is possible that enzymes capable of hydrolysing these condensation products are not present in animal tissues.

It was apparent that the use of water-soluble derivatives was not applicable to studies with progesterone.

b) Solubilisation of progesterone in aqueous media

Progesterone can be brought into aqueous solution by various association colloids (Ekwall and Sjoblom, 1949, 1950; Ekwall, Lundsten and Sjoblom, 1951). It has also been reported that progesterone is soluble in aqueous solutions of α-tocopherol phosphate (Diczfalusy and Westman, 1950). Ercoli and Koller (1949) have dispersed progesterone in an aqueous emulsion of brain phospholipids. Using sodium lauryl sulphate, sodium cholate and polyoxyethylene sorbitan monolaurate ("Tween 20"), Diczfalusy, Ekwall, Sjoblom and Westman (1951) were able to prepare aqueous solutions containing up to 5 mg. progesterone per ml.

The action of synthetic detergents on biological systems has been reviewed by Putnam (1948); in general, ionic detergents exert an inhibitory action on enzymes. Non-ionic detergents on the other hand are not antibacterial nor do they react with proteins nor inhibit some enzymes. Indeed, Archibald (1946) employed "Tween 20" as a substrate in his studies on lipases and Walker (1951) has

used "Tritons" to solubilise β-glucuronidase. Thus it seemed that aqueous solutions of progesterone in non-ionic detergents might be employed for in vitro studies.

Bischoff and Katherman (1948) and Bischoff and Pilhorn (1948) have studied the state and distribution of steroid hormones in biological systems. In the course of their work they determined the solubility of progesterone and other steroids in a variety of aqueous media. Their results for progesterone are given in Table II.

Table II

Solubility of progesterone in aqueous media

(Bischoff and Pilhorn, 1948)

Medium	Equilibration time (hr.)	pH	Solubility (µg. per ml.)
Water	140	-	11.4
0.1% $NaHCO_3$	140	9.1	11.4
3% Bovine serum albumin	12-32	5.3	107
	38	7.4	140
	45	8.1	212
Rabbit serum	24	8.2-8.5	342
	48		440
	72		443

At first sight, therefore, plasma and serum seemed to be suitable media for in vitro studies. Serum is preferable since the presence of an anti-coagulant is more likely to affect enzyme systems than is the absence of fibrinogen. The use of serum for in vitro work has been discussed by Umbreit, Burris and Stauffer (1949) and by Krebs (1950). Their main criticisms are that serum is a non-reproducible medium since the concentration of important constituents such as glucose, pyruvate, acetate and other tricarboxylic acid cycle acids, may vary from sample to sample. These constituents may undergo gross changes during incubation with tissue and the serum might then be converted into a "non-physiological" medium. The bicarbonate content of serum introduces complications in respirometry studies. Serum has been used, however, in a number of in vitro investigations, e.g. MacLeod and Rhoads (1940); Friend and Hastings (1940); Warren (1944, 1947). Clark and Kochakian (1947) employed rabbit serum to disperse testosterone added to rabbit liver slices. It was felt, therefore, that the use of serum solutions of progesterone might prove of some value in the present studies.

During these investigations, the solubility of progesterone in various media was

determined. The methods used and the results obtained are described in the experimental section below.

v) Determination of the solubility of progesterone in aqueous media

a) Materials and Methods

Blood was collected from rabbits (of either sex) sacrificed in the laboratory from time to time. During retraction of the clot, the pooled blood was placed in the refrigerator, The serum was decanted off, centrifuged at 2°, sterilised by ultra-filtration and stored in sterile bottles until required.

Sterile ox serum was supplied by the Bacteriology Department. Bovine serum albumin (3%, w/v) was prepared from 30% bovine serum albumin (Armour Laboratories; Armour & Co.) by dilution with 'phosphate saline', pH 7.4.

Human serum and human serum albumin were obtained from the Regional Transfusion Service (Royal Infirmary, Edinburgh). The serum albumin was described as "containing 24.5% (w/v) albumin at pH 7.2 in 0.2M-$NaHCO_3$ plus a little acetate buffer". The concentration of the albumin was adjusted to 3.2% (w/v) by addition of 'phosphate saline' pH 7.4. The solution was found to be at pH 7.8 and so was adjusted to pH 7.4 by addition of 2N-HCl.

Solutions (1%, w/v) of "Triton X 100" and "Triton A 20" (Rohm and Haas Co., Philadelphia, U.S.A.) (kindly supplied by Dr. A.B. Roy) were prepared in 'phosphate saline', pH 7.4.*

"Neutralised serum" (Friend and Hastings, 1940) was used in all serum experiments. After adjustment to pH 3.0 with 2N-HCl, the serum was subjected to gentle suction at a slow-running water pump for 10 min. with continual shaking of the flask. The pH was then adjusted to 7.4 by addition of 2N-NaOH.

Before addition to the equilibration flask, progesterone was finely dispersed by shaking for 12 hr. in a small test-tube containing two glass beads, 2 ml. water and a "speck" of crude ox-bile. Sterility during equilibration was maintained by addition to the flask of two drops of chloroform. The vessels were tightly closed with ground glass stoppers and gently shaken in a water bath at 37°.

* "Tritons" are a series of non-ionic polymeric surface-active agents based upon alkyl phenols made water soluble by interaction with an alkylene oxide, (Bock and Rainey, 1948).

At the end of the equilibration period protein solutions were treated in the manner described by Bischoff and Pilhorn (1948). Progesterone was determined by the Callow, Callow and Emmens (1938) modification of the Zimmermann (1935) method for the estimation of ketosteroids. In the case of the "Tritons", equilibrated suspensions were filtered at 37°. From this filtrate, 1.0 ml. volumes were added to 2 ml. ethanol and the turbid solutions so obtained filtered. The paper and precipitate were washed 3 x 2 ml. ethanol and the combined filtrate and washings evaporated to dryness in a stream of air. Removal of the detergent in this way was found necessary because the turbidity caused by the "Tritons" interfered in the colour estimation. By the method described "blank" values were negligible. In all filtrations Whatman No. 42 papers, washed 2 x 5 ml. boiling water and 3 x 5 ml. boiling ethanol and then dried at 60°, were used. Colour density was measured in an E.E.L. Colorimeter (Evans Electroselenium Ltd.) against an Ilford Filter No. 624. Readings were converted to weights of progesterone by reference to a "calibration curve". All estimations were carried in duplicate: duplicates were found to vary within the range ±2.5%.

Table III. Solubility of progesterone in aqueous media

(All determinations made at pH 7.4; values in parentheses refer to results of Bischoff et al., 1948)

Medium	Equilibration time (hr.)	Solubility (mg./ml.)
Rabbit serum	48	0.570 (0.44 at pH 8.3
	72	0.58 (0.443 at pH 8.3)
Ox serum	0	0.004
	24	0.565
	48	0.565
	72	0.680
3% ox serum albumin	0	0.004
	42	0.220 (0.212 at pH 8.1)
Human serum	50	0.635
3.2% human serum albumin	50	0.602
1.0% "Triton X 100"	24	0.350
	48	0.365
1.0% "Triton A 20"	24	0.100
	48	0.100

b) Results

The results obtained are tabulated in Table III.

c) Discussion

The values obtained for the solubility of progesterone in rabbit serum and in bovine-serum albumin agree reasonably well with those obtained by Bischoff and Pilhorn (1948). A surprising feature of the results reported here is the indication that the bulk of the solubilising power of human serum appears to be associated with the albumin fraction whereas ox serum has three times the solubilising power of 3% bovine serum albumin. Of interest is the fact that "Triton A 20", which is non-haemolytic (Glassman, 1951) and does not solubilise β-glucuronidase also has a lower solubilising power for progesterone than "Triton X 100" which is haemolytic and does solubilise β-glucuronidase (Walker, 1951).

The results indicate that rabbit, bovine and human sera and human serum albumin are able to solubilise approximately 0.5 mg. of progesterone per ml. while bovine serum albumin and "Triton X 100"

can solubilise about half this amount of steroid. The application of these solutions to the in vitro metabolism of progesterone is described below.

vi) Incubation of protein and detergent solutions of progesterone with rat and rabbit liver and rabbit uterus

Grant et al. (1950) have shown that their method for the determination of pregnanediol in tissue preparations is reasonably satisfactory for as little as 0.5 mg. of pregnanediol in the presence of 250 mg. of liver slices. It was suspected that the presence of serum and increased amount of tissue in the homogenates to be used might not be conducive to quantitative recoveries of the steroid in the experiments contemplated. A series of recovery experiments was therefore carried out in order to determine the lower limit of pregnanediol recovery under the conditions to be employed.

Known amounts of pregnanediol in a minimum volume of ethanol were added to a series of flasks containing 1.0 ml. (350 mg. tissue) rat liver homogenate and 4.0 ml. rabbit serum; 5.0 ml. of 10% KOH (w/v) were immediately added and the flasks processed.

Results

	Pregnanediol added (µg.)	"Pregnanediol" recovered (corrected for "blank") (µg.)	(%)
Experiment I	83	3	3.7
		1	1.2
	335	173	52
		189	56
	670	503	75
		501	75
Experiment 2	100	6	6
		10	10
	300	167	56
		174	58
	600	480	80
		472	78

Though recoveries were very low at 0.1 mg. levels it was felt that pregnanediol would be detectable in incubation experiments provided that 25% of the added progesterone (2.0 mg.) were to undergo conversion.

a) Incubation of pregnanediol in serum solution with rat liver homogenate

Since it is known that liver preparations will metabolise pregnanediol as the water-soluble dihemisuccinate (Grant et al. 1950), it was decided

to attempt to demonstrate the destruction of the "free" steroid by liver in the presence of serum.

Because of shortage of rabbit serum, ox serum was used in this experiment. Pregnanediol (7.0 mg.) and 1.0 ml. water were shaken with a little crude ox bile in a small tube. After 12 hr. shaking the suspension was added to 28 ml. "neutralised" ox serum in a glass-stoppered flask. The mixture was sterilised by addition of a drop of chloroform and incubated for 48 hr. at 37°. In order to determine the effect of this treatment of the substrate on the pregnanediol metabolising system, a solution of pregnanediol dihemisuccinate (PDHS) in serum was prepared in the same way. This was treated in the same manner as the saline solution of PDHS used by Grant et al. (1950) in incubation experiments. Each flask contained 4.0 ml. of serum solution of steroid, 1.0 ml. of rat liver homogenate (containing 300 mg. tissue) and 5.0 mg. DPN and 35 mg. nicotinamide.

The amount of pregnanediol in the serum suspension was determined by treating 4.0 ml. portions with 10% KOH (w/v) followed by the continuous ether extraction and "precipitation" stages of the pregnanediol determination method. While not

expected to give a true value for the amount of steroid present in the serum, it was felt that the value obtained would be of sufficient accuracy to allow comparison to be made between recoveries from incubation and "control" experiments.

Results

	"Pregnanediol" recovered (corrected for "blank")			
	After incubation		From "controls"	
	(µg.)	(%)	(µg.)	(%)
Free pregnanediol in serum	570	86	660	99
	505	76	665	100
Pregnanediol in serum added: 665 µg.				
PDHS in serum	545	79	650	94
	515	75	635	92
Pregnanediol in PDHS added: 690 µg.				

It is apparent that pregnanediol is metabolised by rat liver in the presence of ox serum when added as the free steroid or as PDHS. It seemed possible, therefore, that conversion of progesterone to pregnanediol might occur under the same conditions.

b) Incubation of progesterone in ox serum solution with rat liver homogenate

Ox serum (4.0 ml.) containing approximately 0.66 mg. progesterone/ml. was incubated with 2.0 ml.

volumes of rat liver homogenate (600 mg. tissue/2 ml.) with 5.0 mg. DPN and 35 mg. nicotinamide added. The gas phase in one set of flasks was oxygen and in the other, nitrogen. Incubations were for 2 hr. at 37°.

Results

	µg. "Pregnanediol" formed (corrected for "blank")	
	After incubation	From "controls"
Gas phase: O_2	6	0
	8	0
N_2	8	1
	11	0

c) Incubation of progesterone in rabbit serum solution with rabbit uterus

Three ml. volumes of a rabbit serum solution of progesterone (approximately 500 µg./ml.) were incubated with 0.5 g. minced rabbit uterus, 5.0 mg. DPN and 35 mg. nicotinamide. The rabbit was an immature female. One set of flasks was incubated in oxygen, the other in nitrogen, for 2 hr.

Results

	µg. "Pregnanediol" formed (corrected for "blank")	
	After incubation	From "controls"
Gas phase: O_2	2	1
	2	0
N_2	6	1
	0	0

d) Incubation of progesterone in "Triton X 100" solution with rabbit uterus and rabbit liver

A solution of progesterone (approximately 400 µg./ml.) in "Triton X 100" (1% w/v in 'phosphate saline') was obtained by equilibration for 50 hr. at 37°. Three ml. volumes of this solution were incubated with 0.5 g. minced uterus or 0.5 g. liver slices. No co-factors were added and the gas phase was oxygen in all flasks.

Results

	µg. "Pregnanediol" formed (corrected for "blank")	
	After incubation	From "controls"
Uterus	3	0
	2	2
Liver	7	3
	4	3

vii) Discussion

The results obtained indicate that no appreciable amount of pregnanediol is formed as an end-product when progesterone is incubated with animal tissues *in vitro*. Such conversion as might occur, does not appear to be increased by employing solubilised preparations of the steroid. Wiswell *et al.* (1953) have also failed to demonstrate pregnanediol formation after incubation of progesterone with rat liver. That tissue preparations themselves have sufficient solubilising power to allow steroids to be made available to the enzyme systems involved in metabolism, is indicated by the results of the experiments reported in Section II. In these experiments, progesterone was added to tissue as a solution in propylene glycol.

The acquisition of an ultraviolet spectrophotometer by the Department then provided a means whereby small quantities of progesterone could be accurately determined. It was decided, therefore, to study the metabolism of progesterone by attempting to demonstrate destruction of the steroid by tissue preparations.

viii) Summary of Section I

a) Aqueous suspensions of progesterone and pregnanolone have been incubated with rabbit uterus and rat liver. No significant amount of pregnanediol was detected.

b) A review of methods used to solubilise steroids indicated that solutions of progesterone in protein or non-ionic detergent preparations might be of value in the study of progesterone metabolism.

c) The solubility of progesterone in a variety of aqueous media has been determined.

d) Protein and detergent solutions of progesterone have been incubated with rat and rabbit liver and rabbit uterus. No significant amount of pregnanediol was formed.

SECTION II

THE METABOLISM OF PROGESTERONE BY RABBIT AND RAT LIVER

I. INTRODUCTION

The failure to demonstrate the formation of pregnanediol when progesterone was incubated with tissue indicated that, under the conditions employed:-

i) no significant amounts of pregnanediol were formed, or if formed, were being metabolised further,

ii) no appreciable amount of progesterone was being metabolised,

or iii) progesterone was being converted to metabolites other than pregnanediol.

In view of the ability of liver preparations to metabolise adrenal corticosteroids (Schneider and Horstmann, 1951: 1952), testosterone (Samuels *et al.* 1947) and oestrogens (Cantarow, Paschkis, Williams and Havens, 1952) it seemed that iii) was the most likely explanation. This view received support from the results of *in vivo* experiments with rabbits and rats which indicated that progesterone metabolism must be both rapid and continuous in these animals. Haskins (1950) administered progesterone intravenously to rabbits and found that all the steroid disappeared from the blood within 30 min. of injection. No plasma progesterone was detected at any time during 3 days following intramuscular

injection of the steroid into a rabbit. Butt, Morris, Morris and Williams (1951) found that 95% of intravenously administered progesterone was removed from the circulation of normal rats within 5 min. of injection. Recovery from rats with ligated livers was only slightly higher than in normal rats.

It was therefore decided to study progesterone metabolism by demonstrating disappearance of the steroid after incubation with liver. Such an investigation required a method suitable for the determination of progesterone in the presence of incubated tissue preparations. A study of the methods employed by other workers in this field revealed a number of features which were distasteful to the author. These features may be illustrated by reference to two typical examples.

Samuels (1947) described a method for the determination of testosterone which was later adapted by Wiswell et al. (1953) to the study of progesterone metabolism in vitro. The main steps in this method are:-

i) Coagulation of proteins by boiling the reaction mixture

ii) Batchwise ether extraction of the aqueous residue

iii) Adsorption chromatography on alumina

iv) Solvent partition

v) Determination of unchanged steroid by ultraviolet spectrophotometry.

The method is tedious, requires a large number of vessels and is not highly specific.

Hayano and Dorfman (1953) in their studies on the metabolism of deoxycorticosterone in vitro used a method involving dialysis for 2 days by means of which only 80% recoveries were obtained from control experiments. Recoveries from incubation experments were "adjusted" to give "true" recoveries in terms of 100% recovery from the controls.

In the development of the present method, the following essential features of a reliable analytical method were borne in mind:-

i) Accuracy: quantitative recoveries of progesterone should be achievable over a fairly wide range of concentrations; 100-500 µg. steroid in 3.0 ml. reaction mixture containing up to 500 mg. tissue seemed a reasonable objective.

ii) Practicability: the method was to be such that one operator could carry out a reasonable number of determinations in a reasonably short time. (A minimum of eight in two days was the figure set).

iii) Specificity: the final product obtained

at the end of the extraction and purification procedure should permit the specific determination of progesterone by ultraviolet absorption spectrophotometry.

The method eventually developed largely conformed to these requirements.

The technique of partition chromatography appeared eminently suited to the problem and the development of the method therefore resolved itself into the preparation of extracts suitable for application to partition columns. Modifications in the method of Butt et al. (1951) were necessary because of the larger scale of the experiments planned. Partition chromatography was selected in preference to absorption chromatography because of advantages of the former noted by Butt et al. (1951). From a quantitative standpoint, the chief advantage of partition chromatography, is that elution patterns are symmetrical and "tailing", a great disadvantage of adsorption chromatography, is very greatly reduced. Thus, recoveries are more nearly quantitative.

II. METHODS

i) Development of a method for the determination of progesterone in tissue preparations

a) Introduction

Butt et al. (1951) have described a method for the quantitative extraction of progesterone from blood. Determinations were carried out by polarography following partition chromatography. Bush (1952) has prepared from blood, extracts suitable for application to paper chromatograms in his studies on adrenocorticoids. The extremely low concentrations of steroids encountered by these authors necessitated the preparation of highly-purified extracts before chromatography. In the present investigations relatively greater amounts of steroid were involved and so various simplications of the extraction methods of these authors were at first tried. The original procedures, while providing highly purified extracts were rather tedious and scarcely applicable to routine analyses by one person of the many reaction vessels to be employed in the present studies. The simplified extraction methods yielded promising results, particularly ethyl acetate extraction of the aqueous residue

after removal of protein by acetone precipitation. Quantitative recoveries were only occasionally achieved however.

The use of separating funnels was considered a disadvantage for two reasons; rinsing of vessels and funnels caused an inconvenient increase in the volume of liquid to be processed and emulsions which often formed during extraction caused delay. Centrifugation to remove emulsions caused further delay and further increase in the volume of liquid to be handled. Separating funnels were therefore eliminated from the procedure and extractions carried out in glass-stoppered test tubes (Quickfit and Quartz, MF 24-2, standard taper B 19 joint, capacity approximately 30 ml.) Emulsions could then be removed by centrifugation of the extraction vessel thus eliminating the need for transferences from separating funnels to centrifuge tubes.

Transference of liquids to and from the extraction tubes was achieved by means of an adaptor (Figure 1) which was constructed from a standard taper B 19 joint. Into the adaptor could be fitted:-

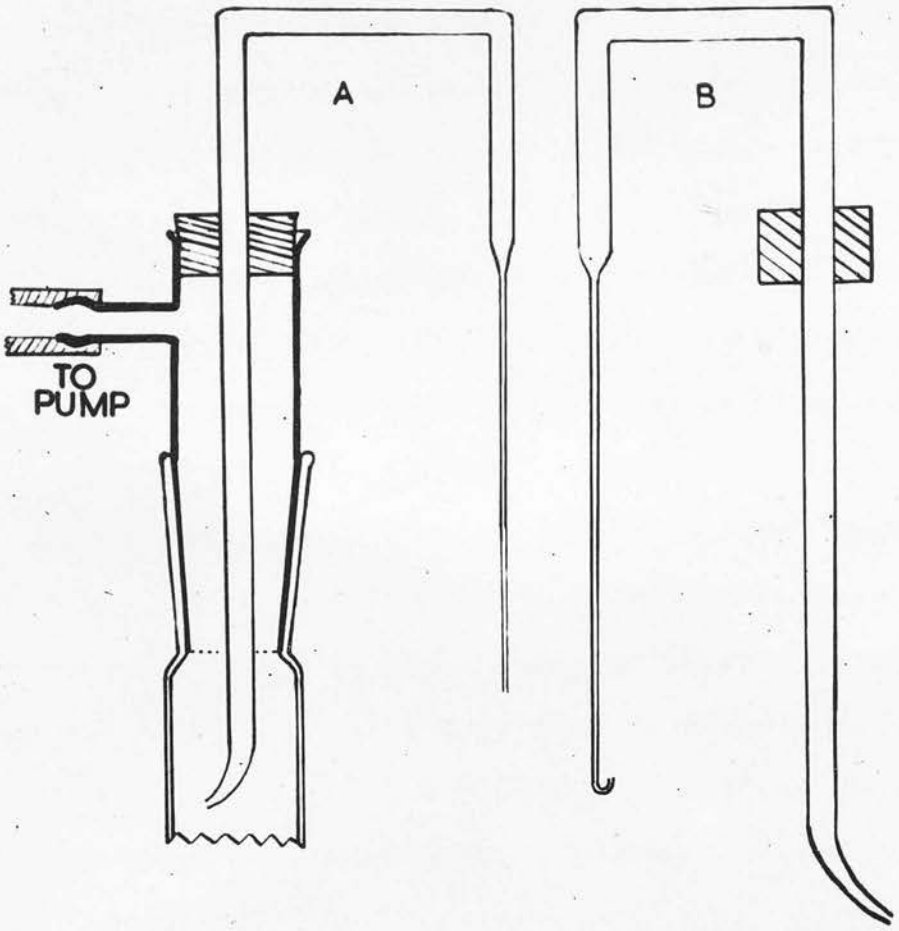


FIG. 1 ADAPTOR & SUCTION TUBES

i) Suction tube A with a straight tip for transference of liquid from the 100 ml. round-bottomed flask (see below).

ii) Suction tube B with a curved tip for transference of the upper layer in the extraction tube back to the 100 ml. flask.

iii) A Hirsch funnel.

After several preliminary experiments the following procedure was adopted.

b) Protein precipitation

At the conclusion of the incubation period 15 ml. cold acetone were added to each reaction vessel. (In preliminary experiments the flasks had been heated in a water bath and the contents filtered while warm. It was found that a very bulky residue was obtained at the final stage and often the partition columns were overloaded and "broke down". Chilling of the flasks to -20° before filtration resulted in a significant reduction in the amount of the final residue, presumably due to the elimination of phospholipids which are only very slightly soluble in cold acetone). After chilling at -20° for 20 min. the contents of the flasks were filtered with suction through paper (Whatman No. I)

supported on a Hirsch funnel, into a 100 ml. round-bottomed flask. (This was achieved by connecting the Hirsch funnel to the flask by means of the adaptor). The precipitate and paper were transferred back to the incubation flask and washed three times more with 10 ml. volumes of hot acetone; each time before filtration the flasks were stored at -20° for 10 min. When slices were used, after two such washings with acetone, the slices were ground up with 10 ml. warm acetone in a glass homogeniser and the resulting powder extracted twice more with 10 ml. volumes of acetone, with chilling at -20° before filtration. The faintly turbid filtrate was concentrated by removing the acetone in a stream of air while the flask was maintained at 45° on a water bath. Evaporation was stopped when about 3 ml. of liquid (mainly water) remained. In preliminary experiments the acetone had been removed by distillation under reduced pressure but spattering which resulted in significant losses of material, was difficult to avoid.

c) Extraction of aqueous residue after removal of acetone

A variety of solvents was tried before a mixture of hexane:chloroform (9:1, v/v) was finally

adopted for the extraction of the aqueous residue. This mixture was considered most suitable for the purpose since it formed the upper layer in a two phase system with water and did not dissolve appreciable amounts of water in contrast to e.g. benzene. (Hexane alone was originally tried but recoveries were not always quantitative. The added chloroform increased the solubilising power of the hexane without causing increased tendency to form emulsions. In the application of this method to the determination of steroids more water-soluble than progesterone it is advisable to increase the solubilising power of the organic phase).

To the aqueous residue in the 100 ml. flask were added 8 ml. of the hexane:chloroform mixture and the contents of the flask refluxed for 5 min. After cooling in a beaker of ice-water, the mixture was transferred to the extraction tube by means of suction-tube A, and the flask rinsed with 3 x 5 ml. hexane:chloroform, 2 x 2 ml. water and finally with 2 ml. hexane:chloroform, each rinsing being transferred to the extraction tube. After centrifugation to clear emulsions, the upper layer was transferred to the original 100 ml. flask (suction-tube B) and the aqueous phase extracted twice more with

15 ml. volumes of hexane:chloroform. The combined extracts in the 100 ml. flask were evaporated to dryness in a stream of air at 45°, removal of the last traces of water being facilitated by addition of a little ethanol. The flasks containing the light-brown gummy extracts were finally dried for at least 1 hr. in vacuo over phosphorus pentoxide at room temperature.

d) Application of extracts to partition columns

After addition of 5.0 ml. of "mobile phase", the flasks were tightly stoppered and left overnight in the temperature-controlled room. (The incubation and extraction procedure require one day to complete and so it was convenient to leave the extract in contact with "mobile phase" overnight in order to ensure complete solution. Preliminary experiments had shown that a minimum of 6 hr. was required to dissolve completely the extracts). To each column was added 0.50 ml. of the extract solution and the walls of the column were washed down with 3 x 0.5 ml. "mobile phase". Each rinsing was allowed to disappear just below the surface of the celite before the next was applied. (During the application of the extract and the rinsing of the column the eluate was collected and

formed part of the first fraction). About 4.0 ml. "mobile phase" were then added to the column, care being taken not to disturb the surface of the celite. A reservoir consisting of a separating funnel with a capillary outlet, was fitted to the column and the rate of flow checked over a period of 10 min. When the rate of flow proved to be outside the required limits, (8-12 ml./hr.) the column was rejected and a fresh one prepared. (Checking of the rate of flow before addition of extract is not valid since the addition of extract may markedly affect the flow rate). The first 10 ml. eluate were rejected and the next 12 ml. ("progesterone fraction") collected for analysis. The solvent was removed in a stream of air and to the dry residue was added a volume of aldehyde-free ethanol estimated to provide a reading of 0.2-0.7 (optical density) on the ultraviolet spectrophotometer for a setting of 240 mμ in a 1.0 cm. cell. (For 500 μg. of progesterone initially added to the incubation flasks, 5.0 ml. ethanol added to each residue provided readings within the required range). The optical density of the ethanol solution was read at 240 mμ. against pure ethanol in the compensating cell and the amount of progesterone present obtained by reference to a "calibration

curve" prepared by measuring the optical densities of solutions of known concentration of progesterone.

The steps in the method may be summarised briefly as follows:

i) Precipitation of proteins by addition of acetone and collection of filtrate in the 100 ml. flask.

ii) Removal of the acetone in an air stream and transference of aqueous residue to the extraction tube.

iii) Extraction of aqueous residue with hexane: chloroform (9:1, v/v) and transference of extract to original 100 ml. flask.

iv) Removal of solvent and drying of residues.

v) Addition of "mobile phase" and after standing overnight, addition of aliquots to partition columns.

vi) Separation of progesterone from extract by partition chromatography.

vii) Determination of progesterone in the eluates from the columns by ultraviolet spectrophotometry.

It should be noted that apart from the incubation flask, only two vessels are used for each extraction. In two full working days, one

operator can complete an experiment involving twelve incubation vessels. No complicated or expensive apparatus is employed and only relatively small quantities of solvents are expended. The method therefore has advantages over the rather tedious procedures used by others.

ii) Column partition chromatography

a) Purification of celite

Celite 535 (Johns Manville & Co.) in shallow trays was heated in a muffle furnace for 8 hr. at 400°. After cooling and transference to a large beaker, the celite was covered with concentrated HCl, A.R., the mixture stirred thoroughly and allowed to stand for at least 12 hr. with occasional stirring. The yellow HCl supernatant was decanted off and the celite transferred to a sintered glass filter with hot tap water. After the bulk of the water had been sucked off, the filter cake of celite was broken up in a large beaker and thoroughly stirred first with hot tap water (10 times) and with distilled water (15 times). The bulk of the water was removed first by decantation then by filtration at the pump. The celite was then allowed to stand overnight in distilled water and was considered to be adequately purified when the filtrate from this final washing was neutral to litmus and contained no chloride (silver nitrate) or iron (ammonium thiocyanate) and when addition of 100 mg. of the celite to 5 ml. distilled water caused no change in pH. The celite was given a preliminary drying by heating

in a porcelain basin with continual stirring. In this dried state it was stored in glass jars but before use it was air-dried at 130° in shallow dishes for at least 12 hr.

b) Preparation of columns

Columns were packed in glass tubes 1.0 cm. in diameter and about 18 cm. long, one end being fitted with a B 14 standard joint and the other being flat glass with small holes (0.5 mm.) regularly placed. Equilibration of solvents and the preparation and running of columns were carried out in a room thermostatically controlled within the range 16 - 18° and usually 17.5 ± 0.5°.

When a large number of columns was to be used at one time, sufficient celite (5 g./column) was weighed into a large specimen jar having a ground-glass stopper and to the celite was added the appropriate amount of stationary phase (1.0 ml./g. celite). The mixture was thoroughly stirred to ensure even distribution and to break up lumps. The jar was then tightly stoppered and left overnight in a temperature controlled room. The next day, the jar was vigorously shaken to ensure completely even distribution of the stationary phase; at this

stage the celite still appeared dry. Sufficient mobile phase was then added to cover completely the celite and a slurry was formed, first by stirring the mixture and then by stoppering the jar and shaking vigorously. (This method of preparing the celite was found to give more consistent results than when 5 g. amounts of celite were weighed separately for each column; evaporation of solvents was reduced to a minimum by the use of a closed vessel; standing the celite overnight ensured even distribution of the stationary phase; a considerable saving in time was also effected). Sufficient of the slurry to pack one column was then transferred to a small beaker and the jar again tightly stoppered. Some of the slurry was transferred to the column which was standing in a test tube containing mobile phase. A packer (Howard and Martin, 1950) consisting of a perforated brass disc (diameter of holes, 1/16") slightly smaller than the internal diameter of the column and attached to a long, centrally fixed, stainless-steel rod was used to pack the columns. The packer was moved rapidly up and down the column, below the surface of the liquid, to distribute the slurry as a fine suspension and to release air bubbles. It was then moved slowly down the column to trap some of the

celite and to compress it into a firm pad, and the column was built up to a height of 10.0 cm. by repeating this operation, each pad being 2 - 5 mm. thick. The first pad, more firmly packed than the rest of the column, effectively sealed the holes at the base of the glass tube and no leakage of celite occurred during the running of the chromatogram. During the packing procedure the celite was always covered with mobile phase and care was taken that no air bubbles were trapped.

Correctly packed columns appeared perfectly uniform and showed no sign of transverse layering; the rate of flow was usually 12 ml./hr. with a 4 cm. head of mobile phase. 10 ml. of mobile phase were allowed to run through each column before application of the sample to be chromatographed.

c) Transference of samples to columns

Three methods were investigated:-

i) That of Butt et al. (1951) in which the sample is dissolved in a small volume (0.1 ml.) of stationary phase and added to a 1 - 2 mm. deep layer of dry celite formed on top of the packed column. The lipid extract of liver obtained in the present investigation was found to be too insoluble

in stationary phase to allow application of this technique.

ii) The lipid extract was dissolved in the minimum volume of ethanol-acetone (50:50, v/v) and the solution transferred by means of a capillary pipette to 20 mg. of dry celite. The latter, on a watch glass, was warmed to evaporate each drop of solution as it was added. When all the solution had been added in this way, the flask which had held the extract was rinsed with small volumes of ethanol-acetone and the rinsings transferred to the celite as before. The mixture of celite and extract was dried in vacuo and then added to the top of the column and pressed down to form a pad approximately 2 mm. thick. Quantitative transferences could not be achieved by this method.

iii) Solution of the lipid extract in mobile phase was found to be the most convenient method. Complete solution was achieved by allowing the extract to stand overnight in contact with mobile phase as described on p. 50.

The "dead volume" of packed columns was determined using the dye Sudan III dissolved in mobile phase and was found to be about 2.5 ml. (cf. Butt et al., 1951).

d) Separation of progesterone from liver extract

Columns 10 cm. in length and of 1.0 cm. diameter were considered to be a convenient size for the separation of about 50 µg. progesterone from one tenth of the lipid extract obtained from up to 500 mg. liver by the method described on p. 47.

In order to determine the optimum conditions for the separation of progesterone from material which might interfere with the spectrophotometric determination of that steroid, a series of preliminary experiments was carried out. Progesterone (500 µg.) was added to the lipid extract obtained from 500 mg. of liver by the method described on p. 47, 5 ml. of mobile phase were added and 0.50 ml. volumes of the solution so obtained were added to columns. Four types of column based on these described by Butt et al. (1951) were used in this first stage. As a rough test for the presence of lipids, the Liebermann-Burchard test for cholesterol was

carried out on the first few ml. eluate; the later fractions were examined for material absorbing specifically at 240 mμ.

Table IV summarises the results obtained with the four systems employed.

Table IV. Retention volume (V_R) of progesterone on columns (10 cm. x 1.0 cm.) with n-hexane as mobile phase and aqueous methanol as stationary phase

Stationary phase % methanol	Stationary phase ml./g. celite	K	V_R(ml.) Calculated	Found
70	0.6	1.98	9.5	10
	1.0		13	15
75	0.6	2.6	11.4	11
	1.0		16	17

For comparison, the calculated retention volumes (V_R) are included in the Table. V_R was calculated from the partition coefficient and dimensions of the column by means of the formula of Butt et al. (1951).

$$K = \frac{V_R}{LA_S} - \frac{A_L}{A_S}$$

A_L : cross-sectional area of mobile phase

A_S : cross-sectional area of stationary phase

L : length of column

V_R : volume of eluate to peak concentration

K : partition coefficient

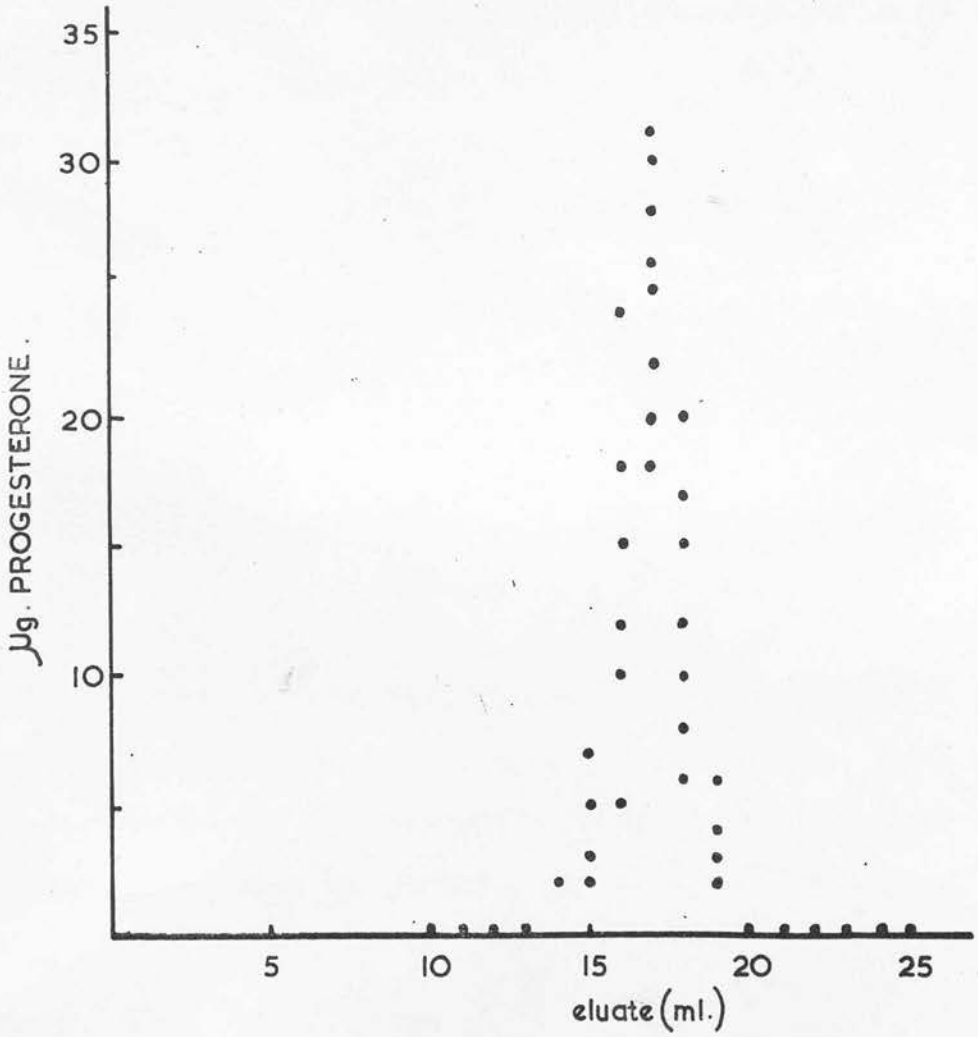


FIG.2. RETENTION VOLUME OF PROGESTERONE.

In order to allow for slight spread of the elution patterns and to minimise any possibility of overlapping of the lipid and progesterone curves, the system in which the retention volume of progesterone was 17 ml. was selected. At this time, eight columns were being used and in order to test the reproducibility of the retention volume of progesterone, the elution pattern of each column was determined. As can be seen from Fig. 2, the elution peak was at 17 ml. and the progesterone appeared in the range 14 - 19 ml. No cholesterol was detectable in the fractions 6 - 10 ml. Analysis of extract to which no progesterone had been added showed that no significant amounts of material absorbing at 240 mμ. were eluted after 6 ml. In quantitative determinations, therefore, the first 10 ml. eluate were rejected and the next 12 ml. collected for progesterone analyses.

iii) The accuracy of the method

The accuracy of the method was checked in a series of experiments involving recovery of known amounts of progesterone from liver slices and homogenates. A propylene glycol solution of the steroid was pipetted into the flasks containing 0.5 g. tissue as slices or homogenate and 3 ml. 'phosphate saline'. Chilled acetone was then added and the flasks worked up immediately.

Results

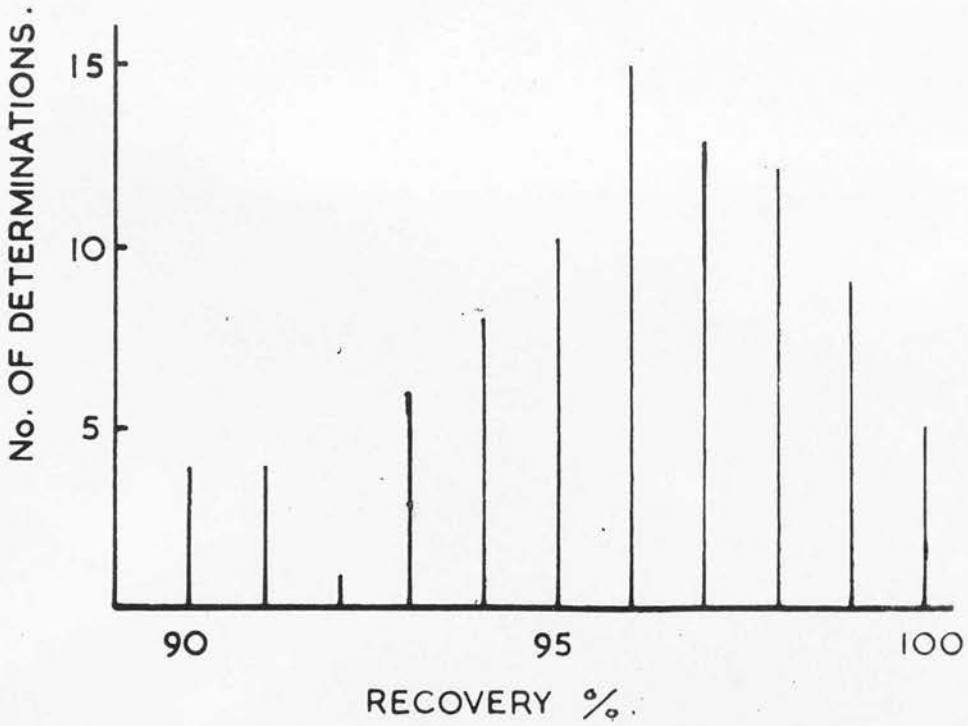
a) Recovery of progesterone from rabbit liver slices

Progesterone added (µg.)	Progesterone recovered (µg.)		(%) (corrected for "blank")
501	506		98
	485		94
	506		98
	485		94
	501		97
	473		91
0 ("Blank")	15)		-
	14)	15	-
	16)		-
	15)		-
200	198		91
	196		90
	196		90
	192		88
0 ("Blank")	16)	15	-
	15)		-

b) Recovery of progesterone from rat liver homogenates

Progesterone added (µg.)	Progesterone recovered (µg.)		(%) (corrected for "blank")
535	530		96
	535		97
	524		95
	524		95
0 ("Blank")	14)		-
	16)	15	-
	14)		-
	15)		-
475	468		95
	467		95
	460		93
	461		92
	14)	15	-
	16)		
194	174		82
	183		87
	194		92
	189		95
0 ("Blank")	14)	14	-
	14)		-
182	170		85
	180		91
	179		91
0 ("Blank")	15)	15	-
	15)		-
86	86		80
	83		67
	83		67
0 ("Blank")	15)	15	-
	15)		-

FIG.3. RECOVERY OF PROGESTERONE FROM "CONTROL" EXPERIMENTS.



The results obtained indicate that the method is reasonably satisfactory for the determination of as little as about 100 µg. progesterone added to 500 mg. tissue in the form of slices or homogenate. At progesterone levels of 75 µg. and below, the method is of little accuracy since the amount of extract which needs to be added to provide a reasonable reading on the spectrophotometer, overloads and causes breakdown of the columns.

Fig. 3 has been constructed by recording the recoveries obtained from the recovery experiments and from the "control" vessels of incubation experiments during the period over which the author has been using the method. It can be seen that the distribution of results is approximating to a normal curve of error with a peak at 96 - 97%.

iv) Specificity of the method

The specificity of the method depends on two factors.

i) The partition coefficient (hence the retention volume on the column) of progesterone in the solvent system, hexane:75% aqueous methanol.

ii) The 240 mμ specific absorption of the α:β-saturated ketonic group in the steroid A ring.

Reference compounds were not available to carry out a series of check experiments but the method was considered to be of adequate specificity as a result of the following argument.

Compounds most closely related to progesterone in structure were considered. Such compounds are:-

i) Pregnane- and 5α-pregnane-3:20-dione

ii) Pregnan-3α-ol-20-one)
iii) 5α-pregnan-3α-ol-20-one) and their C-3 isomers.

These compounds might be expected to have partition coefficients close to that of progesterone in the solvent system used. Indeed, pregnan-3α-ol-20-one was eluted from a column almost simultaneously with progesterone when the solvent system hexane: 70% aqueous methanol was used. However, none of

these compounds absorbs significantly at 240 mμ. and so would not interfere with the estimation of progesterone. The four isomeric "pregnanediols" would not interfere for the same reason, but it was known that pregnane-3α:20α-diol itself was not eluted from the columns used after passage of 50 ml. of mobile phase.

More oxygenated derivatives of progesterone such as 17-hydroxyprogesterone, 11-oxy- or 11-hydroxyprogesterone or "21-hydroxyprogesterone" (11-deoxycorticosterone), while retaining the α:β-unsaturated ketonic group in Ring A, would have partition coefficients in the system 75% methanol:hexane of such magnitude that their retention volumes would be markedly greater than that of progesterone. Hence, interference from such compounds was considered to be unlikely.

Pregn-4-ene-3-one-20-ol might be a possible contaminant of the "progesterone fraction" from the column but it was felt unlikely that this steroid would be encountered since it has not yet been shown to be a metabolic product of progesterone. It can therefore be stated that the method is probably specific for the estimation of progesterone under the conditions employed.

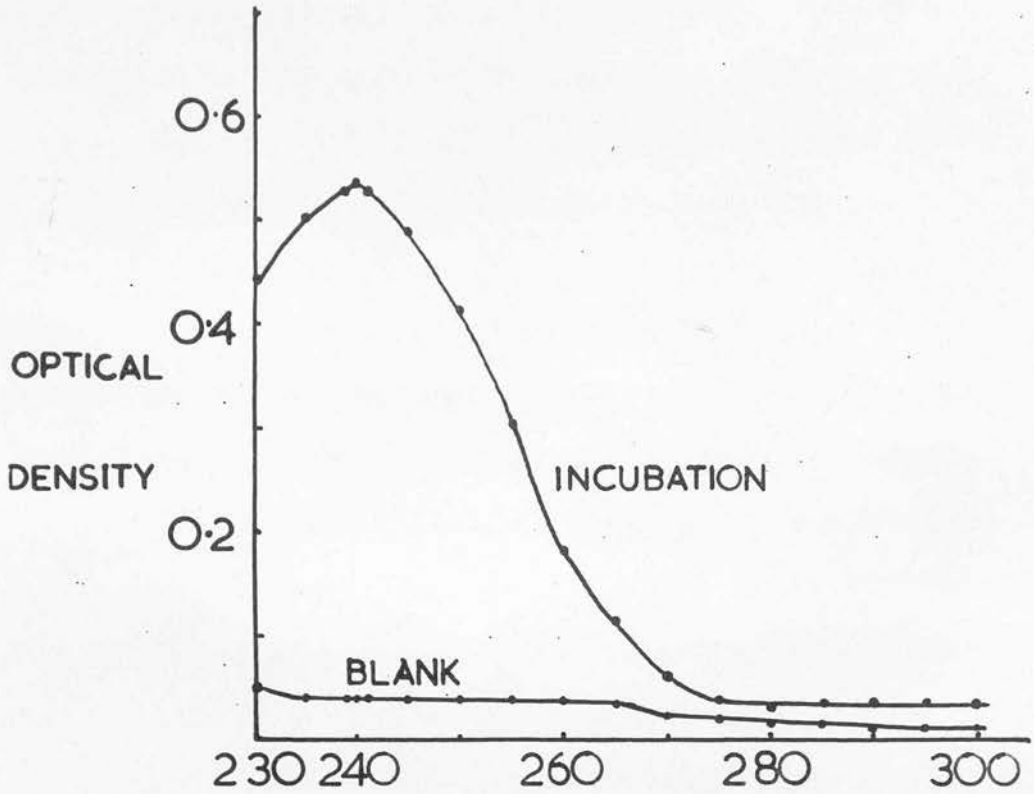


FIG. 4 ABSORPTION SPECTRA OF 'PROGESTERONE' FRACTIONS.

a) The nature of the "blank"

Liver slices (500 mg.) or the equivalent amount of homogenate were incubated with 2.5 ml. of 'phosphate saline'. The mixture was then subjected to the protein precipitation, extraction and chromatographic procedures. The absorption spectrum of the "progesterone fraction" from the column was measured from 230 - 300 mμ. under the conditions normally employed. The method was found to be highly effective in the removal of substances which might interfere with the estimation of progesterone at 240 mμ. In Fig. 4 are shown typical absorption spectra of "progesterone fractions" obtained from blank and incubation experiments. The absorption spectrum of the progesterone recovered from incubation and control experiments was found to be identical with that of a pure solution of the steroid. The blank values were not significantly altered by the presence of added DPN, nicotinamide, etc.

Results

	Blanks expressed as µg. progesterone			
Saline only	12	11	11	12
500 mg. slices in saline	15	9	10	14
500 mg. homogenate in saline	14	16	13	16
500 mg. homogenate in saline plus 3.0 mM-DPN and 80-mM nicotinamide	15	16	15	15

v) Application of the method to the determination of other steroids

The method can be quite readily adapted to the determination of other steroids. The modifications required are:-

i) Development of a suitable solvent system for the partition chromatography of the steroid to be employed.

ii) If the steroid were not amenable to determination by ultraviolet spectrophotometry, a method for the determination of 10-50 µg. amounts would be required.

The author has used the method for the determination of testosterone in the presence of liver mitochondria. For the application of the method to steroids more water-soluble than progesterone, it is recommended that the extraction solvent be modified. The hexane-chloroform mixture used, while ideal for the extraction of progesterone, might possess insufficient solubilising power to extract quantitatively more water-soluble steroids from the aqueous phase.

The method has been successfully applied to the study of 11-deoxycorticosterone and corticosterone metabolism in adrenal mitochondria (Brownie, Grant and Taylor, 1953). In order to obtain quantitative

recoveries of corticosterone, it was necessary to use a benzene-chloroform mixture for the extraction of the aqueous residue. The details of the modification of the method and of the solvent systems employed for the partition chromatograms will be reported elsewhere.

The method has also been applied to the study of progesterone metabolism in relation to citrate synthesis by rat liver mitochondria (Grant and Taylor, 1953) (see Appendix II).

vi) Paper chromatography

Only limited use of paper chromatography was made in the present investigation. It seemed more desirable to concentrate on the quantitative aspects of the problem since it was not certain that the progesterone metabolites would be resolvable or detectable on paper.

Solvent systems of the type proposed by Bush (1952) were employed. The practical difficulties associated with running chromatograms at an elevated temperature were overcome by use of the "chromatocoil" of Schwarz (1952), in which paper strips of about 1 cm. width were accommodated in spiral form in containers which could readily be placed in an incubator. The incubator available was set at 37° and so this temperature was used throughout. Day to day variations in the running of steroids, probably due to slight differences in the width of the strips and paper thickness, were found. Therefore, in each "chromatocoil" jar, two strips were run, one carrying the "unknown" and the other, "standard" steroids.

The reagents used for the detection of the steroids were:-

i) 15% H_3PO_4 (Neher and Wettstein, 1951)

ii) Alkaline m-dinitrobenzene applied by the method of Kritchevsky and Kirk, (1952). Better results were obtained by employing the modification of Savard (1953).

The H_3PO_4 reagent was found to be the most useful for the detection of steroids possessing an α-β-unsaturated ketonic group in Ring A and for highly oxygenated steroids of the "corticoid" type. Hydrogenated derivatives of progesterone ("pregnanediones", "pregnanolones" and "pregnanediols") could not be detected. Ketonic steroids were detected by the second reagent though it was found to be rather insensitive for 20-ketosteroids.

After treatment with colour-developing reagents, the paper strips were found to be very fragile and it was especially difficult to examine the phosphoric acid-treated papers in ultraviolet light. Therefore, after treatment, the strips were placed between two sheets of perspex (ICI, $\frac{1}{8}$ in.) which were then clamped together with strong spring clips. This rigid assembly was transparent to ultraviolet light and the position of fluorescent spots could be marked on the perspex with grease pencil. A scale scratched on the surface of the perspex facilitated the determination of the extent of movement of the spots from the origin.

vii) Ultraviolet spectroscopy

Ultraviolet spectroscopy has become an accepted technique for the qualitative and quantitative analysis of many different types of steroids (Jones, 1948; Friedgood and Garst, 1948). Progesterone itself is a member of that group of steroids, which by virtue of an α-β-unsaturated ketonic group in ring A, exhibit a strong absorption maximum at 240 mμ. At this wavelength the relation between optical density (D) and concentration follows Beer's Law between the limits D = 0.0 - 1.0.

The instrument used throughout was the Unicam Photoelectric Quartz Spectrophotometer, S.P. 500 (Unicam Instruments, Cambridge, Ltd). In order to standardise conditions the following procedures were adopted:

i) For quantitative estimations and preparation of calibration curves at 240 mμ., all measurements were made with a slit-width of 0.60 mm., the galvanometer needle being set to zero by slight adjustment of the sensitivity.

ii) As a check on the stability of the instrument and on the purity of the progesterone fractions from the columns, at frequent intervals, readings were taken at 1.0 mμ. intervals from 235 - 245 mμ

to ensure that the absorption maximum remained at 240 mμ.

iii) For quantitative work it was found that the best working range of the instrument was over the range D = 0.2 - 0.7 and whenever possible, solutions were prepared to give readings within these limits.

iv) Calibration curves were prepared at regular intervals but variations were found to be small. Ethanolic solutions of progesterone were found to be quite stable for periods up to 6 weeks when stored in the refrigerator.

v) Because of the slight difference in translucence of the 10 mm. silica cells supplied, one cell was always used as the compensating cell and the other always contained the solution to be analysed.

vi) Aldehyde-free ethanol was used throughout as the solvent for substances to be analysed. This solvent has the advantage of being transparent from the visual range to 200 mμ.

viii) Miscellaneous

Experimental animals, preparation of tissues and the arrangement of incubation experiments have been described in the General Methods Section.

Addition of progesterone

A weighed amount (torsion balance) of progesterone was dissolved with gentle warming in propylene glycol to provide a solution containing 0.1 mg. steroid per 0.01 ml. This solution was pipetted into reaction vessels by means of an "Agla" micrometer syringe (Burroughs & Wellcome & Co., London).

Two 0.010 ml. volumes of the solution were pipetted into tubes containing 5 ml. ethanol and the solvent was then removed in a stream of air. The tubes were stored *in vacuo* and analysed for progesterone content at the same time as the progesterone fractions from the partition columns. Separate "solvent blanks" were found to be negligible. Thus the amount of steroid added to the reaction vessels could be calculated by reference to a calibration curve.

"Blank" values were found to be invariably low and very consistent. They were therefore not determined routinely but were checked occasionally. A mean 'blank' value of 15 µg. 'apparent progesterone' was used to correct the values found in "incubation" and "control" experiments.

III. THE DESTRUCTION OF PROGESTERONE BY RABBIT LIVER

i) Incubation of progesterone with slices

0.5 g. slices per flask; 1000:1 tissue: steroid ratio.

Results

	Progesterone recovered*	
	(µg.)	(%)
After incubation	99	20
	129	26
	99	20
	75	15
From "controls"	480	96
	447	91
	501	100
	501	100
Progesterone added	501	

Conclusion

Incubation of progesterone with surviving liver slices results in destruction of approximately 75% of the added steroid.

* All recoveries are corrected for "blank".

ii) Comparison of slices and homogenate; effect of citrate

0.25 g. slices or 0.25 g. liver in 1.0 ml. homogenate. Tissue:steroid ratio, 500:1.

Results

	Progesterone recovered (µg.)	(%)
After incubation		
Slices	354	70
	384	74
Homogenate	415	80
	411	80
Homogenate plus 0.001 M-citrate	366	72
	367	72
From "controls"		
Slices	469	94
	483	96
Homogenate	465	91
	480	94
Progesterone added	507	

Conclusions

i) Homogenate is less efficient than slices in causing destruction of progesterone.

ii) Citrate (0.001 M) slightly increases the extent of progesterone destruction by homogenate.

iii) Effect of added DPN and nicotinamide

In the previous experiment it was shown that homogenate is less active in the destruction of progesterone than are slices. A similar loss of activity on homogenising liver has been observed in metabolic studies with oestrogens (Coppedge, Segaloff and Sarrett, 1950), with testosterone (Sweat and Samuels, 1948) and with pregnanediol (Grant *and Marrian*, 1952). It has been attributed by these workers to the rapid destruction of DPN in preparations of broken cells from liver and other organs (Mann and Quastel, 1941). The pH of 7.4 used in the various liver-steroid metabolism studies reported by others and in the present investigation is close to the pH optima of 7.2 (Spaulding and Graham, 1947) and 7.5 (Handler and Klein, 1942) which have been reported for the nucleosidase concerned in the destruction of DPN. This destruction is, however, inhibited by nicotinamide (Mann and Quastel, 1941; Handler and Klein, 1942). It was therefore decided to investigate the effect of DPN and nicotinamide on the metabolism of progesterone by liver homogenate.

Homogenate contained 0.5 g. liver per ml. 1000:1 tissue:steroid ratio. 1.5 mM-DPN and 80- mM nicotinamide in final reaction mixture.

Results

	Progesterone recovered	
	(µg.)	(%)
After incubation		
No added DPN or nicotinamide	442	82
	337	63
	(Loss during processing)	
With added DPN and nicotinamide	82	15
	135	25
From "controls"	510	95
	525	97
Progesterone added	540	

Conclusion

Metabolism of progesterone by rabbit liver homogenate is markedly increased by the addition of DPN and nicotinamide.

IV. THE DESTRUCTION OF PROGESTERONE BY RAT LIVER

For reasons of economy and convenience, the investigations were continued with rat liver. Unless otherwise stated, 0.25 g. liver were incubated with 0.5 mg. steroid: tissue:steroid ratio, 500:1.

i) Incubation of progesterone with slices and homogenate

Results

	Progesterone recovered	
	(µg.)	(%)
After incubation		
Slices	310	62
	330	66
Homogenate	405	81
	390	78
From "controls"		
Slices	490	98
	475	95
Homogenate	485	97
	485	97
Progesterone added	500	

Conclusions

i) Incubation of progesterone with rat liver results in destruction of the steroid.

ii) Slices are more active than homogenate in causing disappearance of progesterone.

ii) The effect of added DPN and nicotinamide

Results

	Progesterone recovered	
Experiment I.	(µg.)	(%)
After incubation		
No additions	375	80
	362	76
With 1.5 mM-DPN and	5	1
80 mM-nicotinamide	5	1
From "controls"		
With 1.5 mM-DPN and	480	100
80 mM-nicotinamide	462	98
Progesterone added	475	
Experiment 2.		
After incubation		
No additions	412	79
	387	72
With 0.7 mM-DPN and	25	5
40 mM-nicotinamide	41	8
With 1.5 mM-DPN and	15	3
80 mM-nicotinamide	5	1
From "controls"		
With 1.5 mM-DPN and	525	98
80 mM-nicotinamide	530	99
Progesterone added	535	

Conclusions

i) Metabolism of progesterone by rat liver is very greatly enhanced by the addition of DPN and nicotinamide.

ii) The effect of these co-factors is more marked in rat liver than in rabbit liver.

iii) The effect of pre-incubation of homogenate with and without added DPN and nicotinamide

The previous experiments do not prove that the reduced activity of homogenate compared with slices could be accounted for by destruction of DPN in the homogenate. The following experiment was therefore performed in order to investigate this possibility.

Pre-incubations were for 30 min. Co-factors added after pre-incubation were added as the solids. Total incubation period, with or without pre-incubation was 90 min.

Results

	Progesterone recovered (µg.)	(%)
After incubation		
No additions: no pre-incbn.	425	77
	405	74
No additions: pre-incbn.	485	86
	475	84
Pre-incbn. with 40 mM-nicotinamide	380	69
	360	65
Pre-incbn. with 40 mM-nicotinamide added after pre-incbn.	435	79
	420	76
Pre-incbn. with 3.0 mM-DPN	425	77
	465	82
Pre-incbn. with 3.0 mM-DPN added after pre-incbn.	275	50
	295	53
No pre-incbn., with 3.0 mM-DPN and 40 mM-nicotinamide	115	21
	76	13
From "controls"		
With 0.3 mM-DPN and 40 mM-nicotinamide	535	97
	520	94
Progesterone added	550	

Conclusions

i) Pre-incubation of homogenate results in decreased activity.

ii) This loss in activity is prevented by the addition of nicotinamide before but not after pre-incubation.

iii) Addition of DPN after pre-incubation increases the activity of homogenate but addition before pre-incubation does not.

iv) The destruction of enzymic activity by heat

The homogenate was added to incubation flasks containing boiling 'phosphate saline' and the mixtures were heated for 10 min. in a boiling water bath. The steroid was then added and the flasks incubated.

Results

	Progesterone recovered	
	(µg.)	(%)
After incubation		
No heat treatment	412	79
	387	72
Heat treatment	525	98
	530	99
From "controls"	525	98
	525	98
Progesterone added	535	

Conclusion

The ability of rat liver homogenate to cause disappearance of progesterone is destroyed by heat.

v) The effect of varying DPN and nicotinamide concentrations

Results

	Nicotinamide (mM)	DPN (mM)	Progesterone recovered (µg.)	(%)
After incubation	-	-	390	78
			400	80
	-	1.0	380	76
			390	78
	-	2.0	310	62
			330	66
	-	3.0	260	52
			250	50
	-	4.0	175	35
			180	36
	40	-	170	34
			150	30
	40	0.3	105	21
			90	18
	40	0.7	25	5
			40	8
	40	1.5	15	3
			30	6
From "controls"	40	1.5	485	97
			470	94
Progesterone added			501	

Conclusion

Optimal conditions for progesterone destruction are provided when 0.7 mM-DPN and 40 mM-nicotinamide are present in incubation mixtures containing 250 mg. tissue and 500 µg. steroid.

vi) The effect of pH

Incubations were carried out in 'phosphate saline' adjusted to the required pH. The homogenate was prepared in KCl solution containing sufficient nicotinamide to provide a concentration of 40 mM-nicotinamide in the final reaction mixture.

Results

	pH	Progesterone recovered (µg.)	(%)
After incubation	6.0	348	74
		352	75
	6.5	239	51
		258	55
	7.0	152	32
		188	40
	7.5	158	33
		140	29
	8.0	388	82
		404	86
From "controls"		456	97
		452	96
Progesterone added		468	

Conclusion

The optimum pH for the destruction of progesterone by rat liver homogenate is in the range 7.0 - 7.5.

vii) Incorporation of acid hydrolysis into the extraction procedure

The method of extraction of incubated mixtures would fail to recover water-soluble conjugates of progesterone which might be formed during incubation. In order to investigate the possibility that conjugation was responsible for the disappearance of progesterone, the procedure was modified to include an acid hydrolysis.

After precipitation of proteins and removal of acetone, 5 drops of concentrated HCl were added to the aqueous residues and the flasks were heated for 10 min. in a boiling water bath. Before extraction with hexane-chloroform, the mixtures were neutralised by the addition of solid sodium carbonate.

(40 mM-nicotinamide and 0.7 mM-DPN in final reaction mixtures).

Results

	Progesterone recovered (µg.)	(%)
After incubation		
No acid treatment	40	8
	40	8
After acid treatment	40	8
	40	8
From "controls"		
No acid treatment	464	96
	460	95
After acid treatment	460	95
	460	95
Progesterone added	484	

Conclusion

Acid hydrolysis does not increase the amount of progesterone recovered.

viii) Varying incubation time

Pairs of flasks (nicotinamide concentration, 40 mM) were incubated for varying periods of time.

Results

	Incubation time (min.)	Progesterone recovered (µg.)	(%)
After incubation	15	350	66
		335	63
	30	285	54
		280	53
	45	250	47
		240	45
	60	175	33
		165	31
	75	165	31
		165	31
	90	170	32
		157	29
From "controls"	90	500	93
		515	96
Progesterone added		535	

Conclusion

Progesterone destruction is rapid and continues for about 60 min.

ix) The effect of anaerobic conditions

a) Nitrogen

In this experiment, one pair of flasks was gassed with O_2 and another pair with N_2. The homogenate was prepared in KCl-nicotinamide; nicotinamide concentration in reaction mixture, 40 mM. The nitrogen was purified by passage through "Fieser's solution" (1929).

	Gas phase	Progesterone recovered	
		(µg.)	(%)
After incubation	O_2	100	20
		85	17
	N_2	85	17
		50	10
From "controls"	N_2	475	95
		485	97
Progesterone added		500	-

b) Evacuation of reaction vessels

Incubations were carried out in Thunberg tubes, one pair of which was evacuated by applying suction from a vacuum oil pump for 5 min.

	Progesterone recovered	
	(µg.)	(%)
After aerobic incubation	175	33
	165	31
After anaerobic incubation	165	31
	185	35
From "controls"	500	93
	515	96
Progesterone added	535	-

Conclusion

Destruction of progesterone is not decreased in the absence of oxygen.

x) The effect of citrate

The homogenate was prepared in KCl with no added nicotinamide.

	Citrate concn. (mM)	Progesterone recovered (µg.)	(%)
After incubation	0	395	81
		380	78
	1.0	90	19
		85	18
	2.0	95	19
		85	18
	3.0	65	13
		55	12
From "controls"	3.0	475	98
		475	98
Progesterone added		485	

Conclusions

i) Addition of citrate increases the ability of the system to metabolise progesterone.

ii) The effect appears to be maximal at a citrate concentration of 1.0 mM.

xi) The effect of tricarboxylic acid cycle intermediates, adenylic acid (AMP) and adenosine triphosphoric acid (ATP)

The effect of various members of the tricarboxylic acid cycle with and without added ATP and of AMP was investigated. ATP and AMP were the commercial products of Light & Co.

a)	Progesterone recovered	
	(µg.)	(%)
After incubation		
No additions	350	76
	390	84
With 1.0 mM-fumarate	380	82
	350	76
With 1.0 mM-α-keto-glutarate	420	90
	400	86
With 1.0 mM-malate	390	84
	400	86
With 5.0 mM-AMP	400	86
	380	82
From "controls"	435	94
	450	98
Progesterone added	468	

b)

	"Acid" (1.0 mM)	ATP (0.5 mM)	Progesterone recovered (µg.)	(%)
After incubation	-	-	505	77
			535	81
	-	+	530	81
			525	80
	α-ketoglutarate	-	580	88
			530	81
	α-ketoglutarate	+	505	77
			565	86
	succinate	-	520	79
			570	87
	succinate	+	510	78
			565	86
From "controls"				
	succinate	+	645	98
			635	97
Progesterone added			655	

Conclusions

i) The presence of intermediates of the tricarboxylic acid cycle does not result in increased destruction of progesterone.

ii) ATP and AMP do not influence progesterone metabolism

V. PRODUCTION OF FORMALDEHYDOGENIC MATERIAL AFTER INCUBATION OF PROGESTERONE WITH RABBIT AND RAT LIVER

Preliminary experiments (not reported here) had indicated that formaldehydogenic material (FSS) was produced when progesterone was incubated with rabbit liver. Therefore, a series of more carefully controlled experiments was carried out.

a) Materials and Methods

After protein precipitation and removal of acetone in the normal way, the aqueous residue was extracted with benzene:chloroform (6:1, v/v) instead of hexane:chloroform in order to ensure quantitative extraction of steroids more "polar" then progesterone. After removal of the solvent, the residues were taken up in chloroform and the solution washed 1 x 1/10 vol. 0.1N-NaOH and 2 x 1/10 vol. water. In one experiment a partition between 80% methanol-hexane was introduced but as no significant amount of FSS was removed by the hexane, this stage was omitted in later experiments. (These extractions and washings were carried out in test tubes in the same manner as for the hexane-chloroform extractions

in progesterone determinations). The residues were dissolved in benzene and separate portions used for progesterone analysis, for formaldehyde determinations and for paper chromatography. Formaldehyde was determined by the method described by Patterson and Marrian (1953). Results are expressed in equivalents of deoxycorticosterone (DOC). The colour reaction used to develop the strips from the "chromatocoils" was 15% H_3PO_4 (Neher et al., 1951).

b) Results

i) Rabbit liver

Formaldehyde determinations were carried out on one flask from each pair. One pair of incubation flasks and the control and blank flasks contained 3.0 mM-DPN and 80 mM-nicotinamide. The progesterone was added in propylene glycol. Incubation time, 60 min.

(µg.)

	Progesterone metabolised	HCHO in portion analysed	FSS as DOC	
			Total	Corrected for "blank"
After incubation				
No additions	128	-	-	-
	152	1.6	77	(-13)
With DPN and nicotinamide	440	-	-	-
	452	4.1*	200	110
From "controls"	12	-	-	
	24	1.8	82	90
From "blanks"	-	-	-	
	-	2.2	98	

Paper chromatograms

Solvent system, benzene-50% aqueous methanol, (Bush, 1952, system B_5) and 60:30 hexane:benzene, 80% aqueous methanol.

The residues obtained after incubation with DPN and nicotinamide showed two strong violet spots, R_F values 0.35 and 0.75 and the green spot of

* In this and the following tables of results the asterisk indicates that the tube so marked showed the true "formaldehyde colour". Other tubes were usually brown in colour.

progesterone at the solvent front (R_F = 1.0). The control residue showed only the green progesterone spot (R_F = 1.0) and the blank residue gave no spots.

With system B_3 the violet spots had R_F, 0.6 and 0.9 and the progesterone green spot had R_F = 1.0

ii) Rat liver

a) No DPN was added but the homogenate was prepared in KCl-nicotinamide (final nicotinamide concentration, 80 mM). The progesterone was added in propylene glycol.

(µg.)

	Incubation period (min.)	Progesterone metabolised	HCHO in portion analysed	FSS as DOC	
				Total	Corrected for "blank"
After incubation	15	200	2.2	122	(-40)
		185	3.7*	203	41
	30	250	3.4*	187	25
		245	3.5*	192	30
	45	285	3.5*	192	30
		295	4.6*	253	91
	60	360	3.1*	170	12
		350	4.1*	225	63
From "controls"	60	35	3.5	192	162
		20	2.7	148	
From "blanks"	60	-	2.8	154	
			2.8	154	

b) Because of the high blank values obtained in the previous experiments, presumably due to the presence of propylene glycol, itself formaldehydogenic, the progesterone was added in ethanol henceforth.

i) Incubations were for 45 min.; 40 mM-nicotinamide in reaction mixture.

(µg.)

	Progesterone metabolised	HCHO in portion analysed	FSS as DOC	
			Total	Corrected for "blank"
After incubation	150	6.0*	132	82
	146	3.1	68	18
From "controls"	10	2.9	64)	
	15	1.9	42)	
			)	50
From "blanks"	-	2.0	44)	
	-	2.3	51)	

ii) Incubation period, 60 min., 2.0 mM-DPN and 40 mM-nicotinamide in reaction mixture.

(µg.)

	Progesterone metabolised	HCHO in portion analysed	FSS as DOC	
			Total	Corrected for "blank"
After incubation	420	4.5	82	11
	434	6.0*	110	39
	410	6.5*	115	44
	420	5.9*	104	33
From "controls"	12	4.4	77	)
	16	3.6	66	)
				) 71
From "blanks"	-	4.2	77	)
	-	3.4	64	)

iii) DPN (2.0 mM) and nicotinamide (40 mM) in reaction mixture.

(µg.)

Incubation period (min.)	Progesterone metabolised	HCHO in portion analysed	FSS as DOC	
			Total	Corrected for "blank"
After incubation 60	315	2.0	110	39
		1.6	88	17
120	325	3.5*	192	121
	340	2.5	137	66
From "controls" 60	16	-	-	
	12	2.0	110	) 71
120	10	1.0	55	)
	15	1.0	55	)

Paper chromatography of the residues from rat liver experiments yielded inconclusive results. In most cases progesterone from the "controls" was the only steroid detected.

Conclusion

Incubation of progesterone with rat and rabbit liver results in production of formaldehydogenic material.

VI. THE ISOLATION OF METABOLIC PRODUCTS AFTER INCUBATION OF PROGESTERONE WITH RAT LIVER HOMOGENATE

i) Introduction

In the quantitative, small-scale, experiments already described, 0.25 g. tissue and 500 µg. progesterone were suspended in 3.0 ml. of reaction mixture. To employ these proportions of tissue, steroid and medium in the large scale experiment envisaged would have involved handling unwieldy amounts of liquid. Therefore it was decided to double the amounts of tissue and steroid and to employ the same volume of suspension medium. In order to test the efficiency of this system a small scale preliminary experiment was carried out. Homogenate (1.0 ml.: 0.5 g. tissue), prepared in KCl-nicotinamide (final concentration, 80 mM) was incubated in 2.0 ml. 'phosphate saline' with 1.0 mg. progesterone in 0.10 ml. propylene glycol. Under these conditions approximately 90% of the progesterone was metabolised in 2 hr.

It was considered that 300 mg. of progesterone would be a convenient amount for the isolation

of the metabolic product or products. At a tissue to steroid ratio of 500:1, this amount of progesterone required 150 g. liver (obtainable from 30 rats) and since it was intended to include a "control" (steroid added after incubation) and a "blank" (no added steroid) the large scale experiment was conducted in two parts. In the first part of the experiment, 150 mg. progesterone were incubated with homogenate and a "control" treated in an identical fashion, was run at the same time. The homogenate was prepared in small batches in chilled, nylon-glass homogenisers as described above. The Atomix Blender was not considered suitable for the preparation of large amounts of homogenate in view of the reports by Stern and Bird (1949) and Lambden (1950) that the similar Waring Blendor is capable of inactivating certain enzymes. In the second part, a further 150 mg. of progesterone were incubated and a "blank" was run at the same time. Because of the tedious and time consuming nature of homogenising the liver in small batches, in this part of the experment, the tissue was homogenised in a "Nelco" Blendor, run at half speed for 2.5 min. (A preliminary small-scale experiment had shown that homogenate prepared in this manner was able to metabolise progesterone).

ii) Experimental

a) Preparation of homogenate

Approximately thirty female rats, about 6 months old were killed by decapitation and the livers rapidly excised and chilled in ice-cold, isotonic KCl. The livers were blotted with filter paper and rapidly chopped finely with scissors on chilled watch glasses and weighed into appropriate lots (5 g. for the nylon-glass homogeniser, 25 g. for the blendor in the second part). Homogenisation was carried out in 0.15M- KCl containing 80 mg. nicotinamide per ml. When 150 g. liver had been homogenised the volume was made up to 300 ml. with the KCl-nicotinamide; in all, 100 ml. of this solution were required and so the nicotinamide concentration in the final mixture was approximately 70 mM.

b) Incubation

It was convenient to conduct the incubations in 100 ml. conical flasks; thirty of these were prepared. To each was added 20 ml. chilled 'phosphate saline' and 10 ml. homogenate (equivalent to 5 g. liver). A propylene glycol solution of progesterone

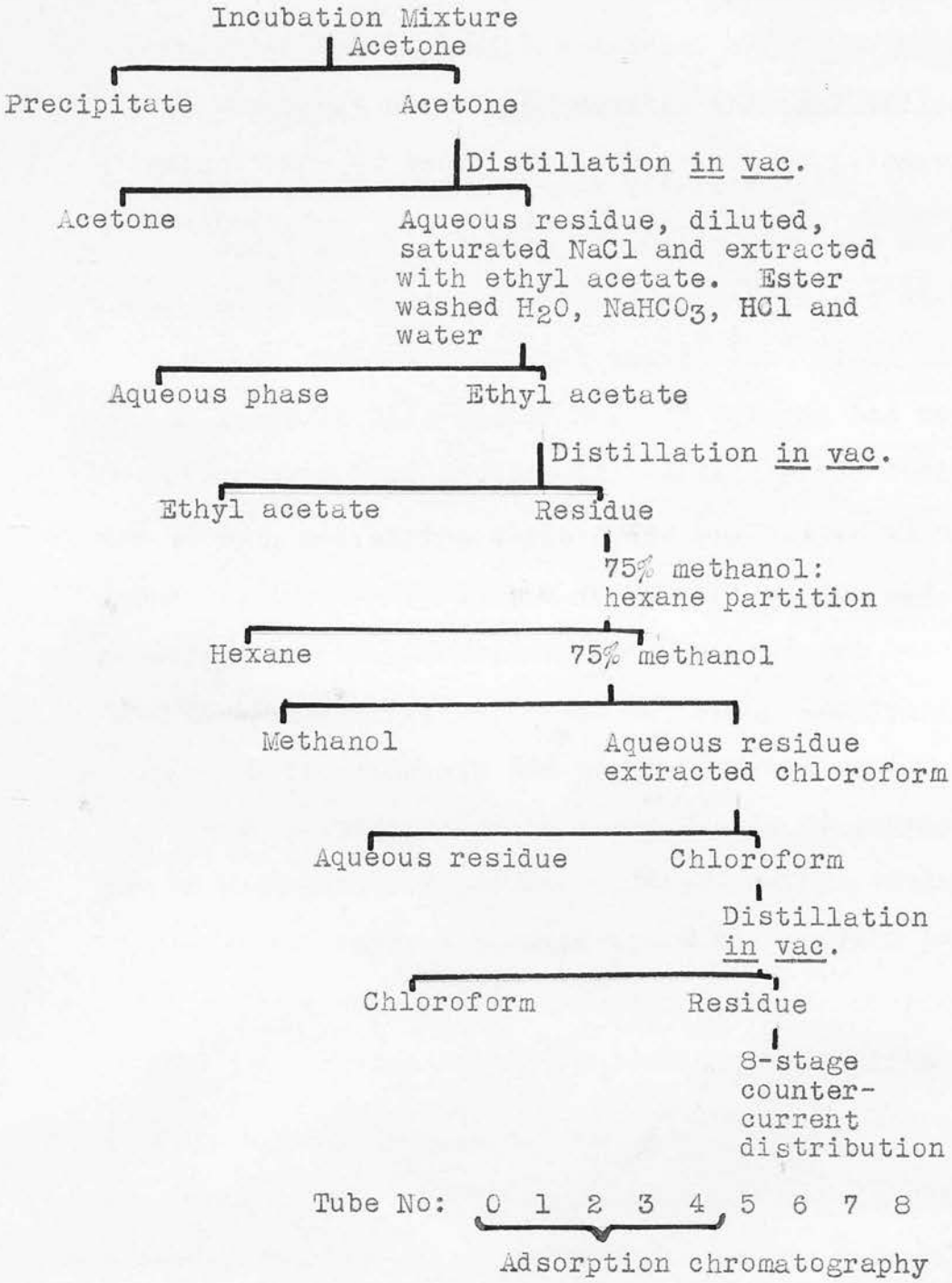


Table V. Summary of extraction and purification procedure

(10 mg./ml.) was warmed to 37° to reduce the viscosity of the solvent and 1.0 ml. volumes were pipetted into the fifteen incubation flasks. Pure solvent (1.0 ml. volumes) was added to the 'control' or 'blank' vessels. The flasks were not stoppered but were shaken quite vigorously during the incubation period of 2 hr.

c) Extraction

(The extraction and purification procedure is shown schematically in Table V).

"Incubations", "control" and "blank" were treated separately in the following manner. At the end of the incubation period, the contents of the flasks were emptied into a large beaker. Each flask was rinsed with 50 ml. volumes of hot acetone until a total volume of 2 l. of acetone had been employed, and the washings added to the beaker. After vigorously stirring, the mixture was allowed to stand overnight at -20°. The yellow supernatant was filtered off and the precipitate returned to the beaker and washed twice with 1 l. volumes of acetone previously chilled to -20°. Removal of the bulk of the acetone from the combined filtrates was achieved by distillation under reduced pressure (in this and

subsequent distillations the bath temperature was kept below 60°). After dilution with 250 ml. of water the aqueous residue was saturated with NaCl and the yellow oil which separated was extracted 4 x 250 ml. ethyl acetate. The ester extract was washed successively with 80 ml. volumes of water, 0.2M-$NaHCO_3$, 0.2N-HCl and finally three times more with water. The final water wash was neutral. Washing was carried out rapidly so that the ester was in contact with alkali or acid for the shortest possible time. The first water and $NaHCO_3$ washes were combined and backwashed with 200 ml. ethyl acetate and the same 200 ml. was used to backwash the HCl and final water washes. After drying over anhydrous Na_2SO_4, the combined ester extracts were evaporated to dryness by distillation in vacuo.

The resultant brown gum was taken up in 300 ml. methanol and 100 ml. n-hexane; 100 ml. water were added and two layers separated, the hexane layer being appreciably yellow. The mixture was transferred to a separating funnel and the distillation flask rinsed into the funnel with 100 ml. 75%-aqueous methanol and 50 ml. hexane. The hexane layer was extracted twice more with 100 ml. volumes of 75%-methanol and the combined aqueous methanol extracts washed with 50 ml. hexane before the bulk

of the methanol was removed by distillation in vacuo.

An equal volume (150 ml.) of water was added and the aqueous residue extracted 2 x 500 ml. chloroform. Washing the chloroform with 100 ml. N-NaOH caused appreciable darkening of colour and the aqueous layer was yellowish-brown. The chloroform was further washed with 100 ml. volumes of water until neutral. After being dried over anhydrous Na_2SO_4 the chloroform extract was evaporated to dryness in vacuo. The weights of the extracts obtained at this stage were as follows:-

Part 1. (Homogenisation in nylon-glass homogeniser).

"Incubation"	0.249 g.
"Control"	0.300 g.

Part 2. (Homogenisation in blendor).

"Incubation"	0.223 g.
"Blank"	0.436 g.

d) Counter-current distribution

The extracts were then subjected to an 8-stage discrete counter-current distribution (Craig and Craig, 1950) carried out in separating funnels using the solvent system 80:20 methanol:water, 80:20 benzene:hexane, in which K for progesterone is 0.25 and for

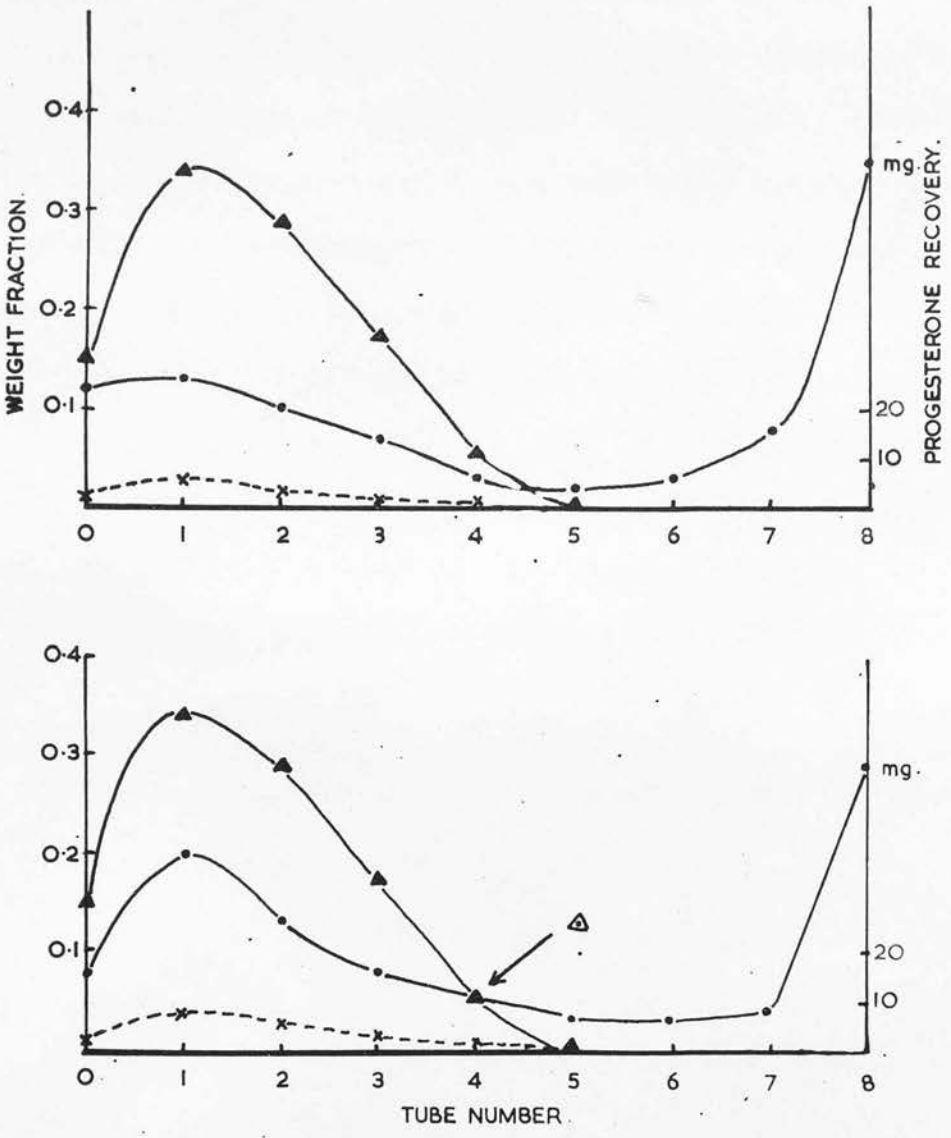


FIG.5. COUNTER CURRENT DISTRIBUTION OF INCUBATIONS FROM PARTS 1 AND 2 OF LARGE SCALE EXPERIMENT.

11-deoxycorticosterone, 1.0. Progesterone analyses were carried out on the residues obtained from each tube. Figs. 5 and 6 show the distribution patterns obtained with the "incubations", "control" and "blank" respectively. The theoretical curve for K = 0.25 is included.

The theoretical curve for K = 0.25 was calculated by expanding the binomial $\left(\frac{1}{K+1} + \frac{K}{K+1}\right)^n$ which gives the fraction of solute in a given tube on completion of the distribution.

The individual terms may be expressed by the formula

$$T_{n,r} = \frac{n!}{r!(n-r)!} \left(\frac{1}{K+1}\right)^n K^r$$

where $T_{n,r}$ = fraction of original material present in the rth after n transfers. K is defined as the ratio of the solute in the upper phase to that in the lower, since in the extraction trains usually employed, the upper is the mobile phase. When the distribution is carried out in separating funnels, the lower, is the mobile phase and so K = 0.25 for the progesterone represents the ratio,

$$\frac{\text{concn. in aqueous methanol}}{\text{concn. in benzene-hexane}}$$

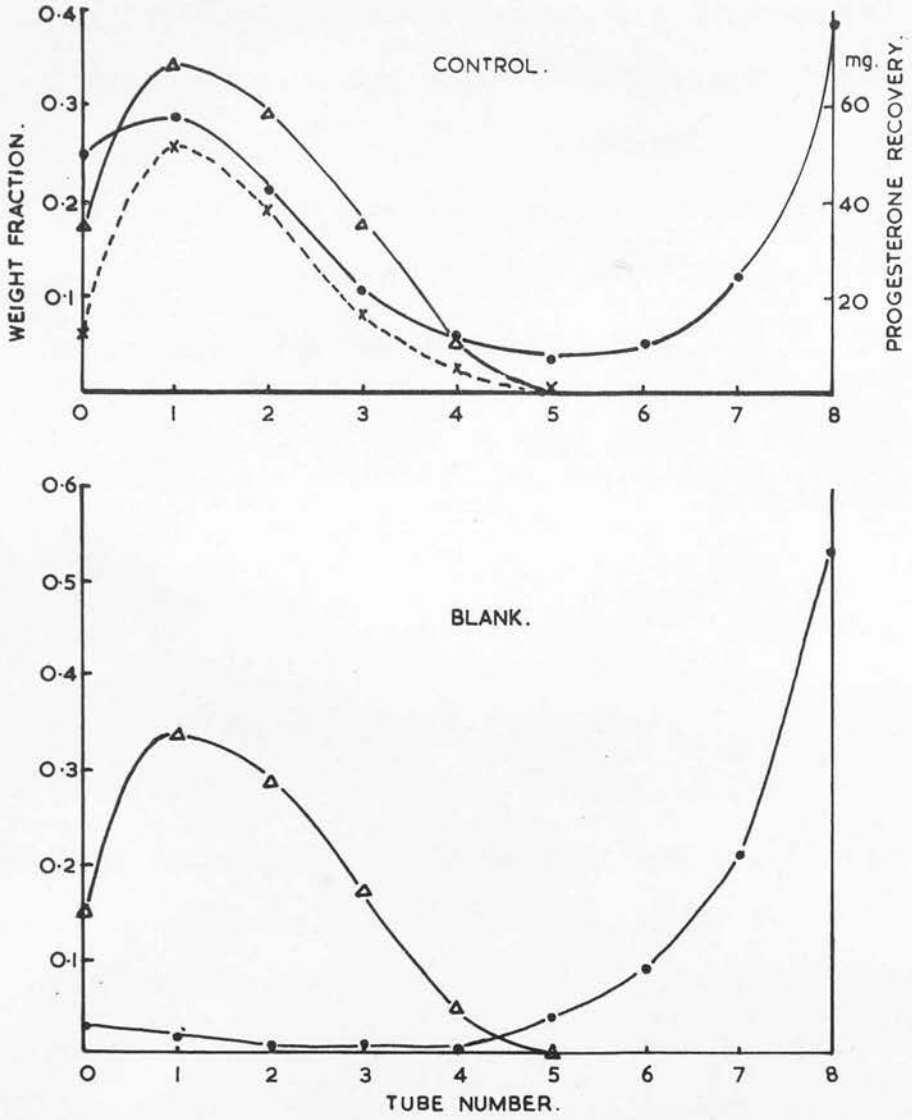


FIG.6. COUNTER CURRENT DISTRIBUTION OF "CONTROL" AND "BLANK" OF LARGE SCALE EXPERIMENT.

The solvent system 80:20 methanol-water, 80:20 benzene:hexane was selected in preference to the aqueous methanol-hexane system because of the greater solubility of the extract in the former.

The curves for the extracts are expressed in terms of the weight fraction per tube while the determined curve for progesterone is expressed as mg. per tube. It can be seen that little progesterone remained after incubation.

A high degree of purification was achieved in this way: the residues from tubes 0 - 4 were semi-crystalline and almost perfectly white. Melting points were not sharp, however, and crystallisations attempted with a variety of solvents were unsuccessful.

It was apparent from the distribution patterns that the bulk of the metabolic products was present in tubes 0 - 4 and that no appreciable amounts of substances as highly oxygenated as 11-deoxycorticosterone had been formed. This suggested that the products consisted almost entirely of hydrogenated derivatives of progesterone. Since adsorption chromatography had been successively applied to the separation of pregnane derivatives by Dobriner, Lieberman and Rhoads, (1948) it was decided to employ their technique at this stage.

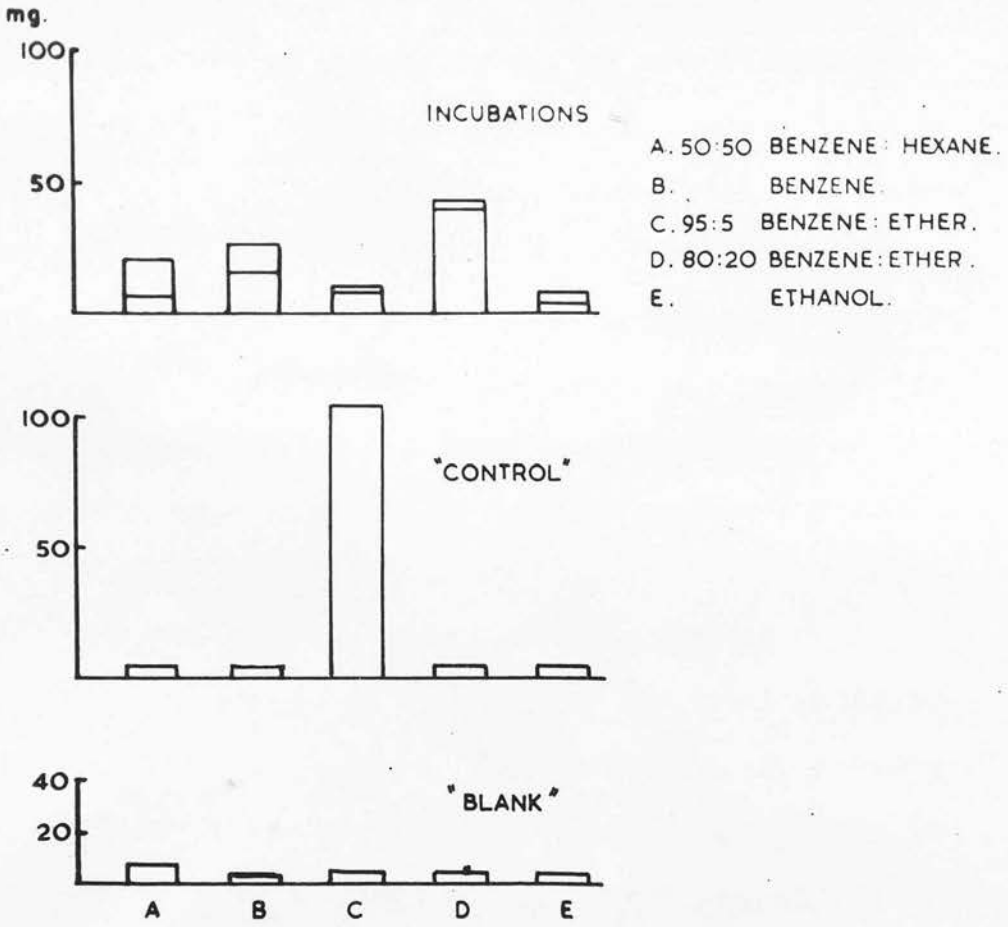


FIG.7. ELUTION PATTERNS FROM ADSORPTION CHROMATOGRAMS.

e) Adsorption chromatography

The residues from tubes 0 - 4 were combined and chromatographed on thirty times the weight of alumina (Peter Spence; activity II after Brockman). The material dissolved in a minimum volume of 50:50 benzene:hexane was applied to the column and the following fractions collected: 5 x 10 ml. 50:50 benzene:hexane; 10 x 10 ml. benzene; 5 x 10 ml. 95:5 benzene:ether; 5 x 10 ml. 80:20 benzene:ethanol and finally 25 ml. ethanol.

The elution patterns of the columns are shown in Fig. 7.

Fraction A: The residues from this fraction were dark brown gums and were rejected.

f) Isolation of 5α-pregnane-3:20-dione

Fraction B: In the case of the incubation experiments a total of 40 mg. of semi-crystalline material, m.p. 197 - 199.5° was obtained. After two crystallisations from acetone, this material yielded 30 mg. of shimmering white plates, m.p. 199 - 200° (Substance "X"). A comparison of the properties of this substance with those of 5α-pregnane-3:20-dione provided proof that Substance "X" was 5α-pregnane-3:20-dione. (Table VI).

Table VI. Comparison of substance "X" with 5α-pregnane-3:20-dione

	"X"	5α-pregnane-3:20-dione	
		Found	Reported
M.p.	199-200°	199-200.5°	200-201° (a)
Mixed m.p.	198.5-199.5°		
C,H Analysis	Found		Calc. for $C_{21}H_{32}O_2$
	C, 79.5, 79.5 H, 10.5, 10.4	C, 79.6, 79.5 H, 10.3, 10.3	C, 79.7 H, 10.2
$[\alpha]_D$ in EtOH	+110±1.5° at 19.5° (c, 0.537)	+108±2.8° at 19° (c, 0.5897)	+117±4° at 24° (a) (c, 0.255)
Disemicarbazone			
M.P.	>300°	>300°	>300° (b)
Mixed m.p.	>300°		

a) Lieberman et al. (1947)
b) Kyle and Marrian (1951)

The residues from the control and blank experiments yielded no crystals.

Fraction C: It was apparent from the elution patterns that this fraction contained progesterone. Recrystallisation of this fraction from the control experiment yielded 84 mg. progesterone and from the incubation experiments a total of 12 mg. was obtained. The progesterone was identified by m.p., mixed m.p., absorption spectrum and by paper chromatography.

g) Isolation of 5α-pregnan-3α-ol-20-one

Fraction D: Only the incubation experiments yielded any appreciable material in this fraction. The residues from both incubation experiments were combined and rechromatographed on alumina. The material was added in benzene and the column eluted with the following series of solvents, 5 ml. fractions being collected; 30 ml. benzene, 35 ml. 1:9 ether:benzene, 15 ml. 2.5:7.5 ether:benzene, 25 ml. 5:5 ether:benzene, 20 ml. 9:1 ether:benzene and finally with 25 ml. ethanol.

Two main fractions were obtained; the 1:9 ether:benzene fractions yielded 25 mg. of a semi-crystalline white solid melting in the range 165 - 172° (Substance Y) and the combined 5:5 ether:benzene

Table VII. Comparison of substance "Y" with 5α-pregnan-3α-ol-20-one

	"Y"	5α-pregnanolone*
M.p.	173-175°	174-175°
C,H Analysis	Found	Calc. for $C_{21}H_{34}O_2$
	C, 79.4, 79.0 H, 10.9, 10.9	C, 79.2 H, 10.8
$[\alpha]_D$ in EtOH	+99.0±1° at 21° (c, 0.524)	+96.6±1° at 25° (c, 0.755)
Acetate		
M.p.	141-143°	140-142°
C,H Analysis	Found	Calc. for $C_{23}H_{36}O_3$
	C, 76.0	C, 76.6
	H, 9.9	H, 10.1

* Lieberman et al. (1948)

fractions yielded 22 mg. of a gum which turned white on being spotted with hexane (Substance Z).

Substance "Y" was recrystallised from aqueous methanol and yielded 20.5 mg. of fine plates, m.p. 173 - 175°. It gave no precipitate with digitonin and mixed with 5α-pregnan-3β-ol-20-one (m.p. 193 - 195°) the m.p. was 152 - 167°. Table VII shows the properties of Substance "Y" compared with those of 5α-pregnan-3α-ol-20-one. No authentic sample was available for comparison but it appears certain that Substance "Y" was 5α-pregnan-3α-ol-20-one.

h) Substance "Z"

Recrystallisation was attempted from a number of solvents but without success and so the material was rechromatographed on alumina; no separation was obtained though the residues were now semicrystalline. The material was eluted from activity II alumina by 1% ethanol in benzene but not by benzene. Fractional crystallisation from aqueous acetone yielded three specimens of silky white needles m.ps. 123 - 130°, 121 - 130° and 105 - 124° respectively. Mixture with androstane-3:20-dione depressed the melting point in each case.

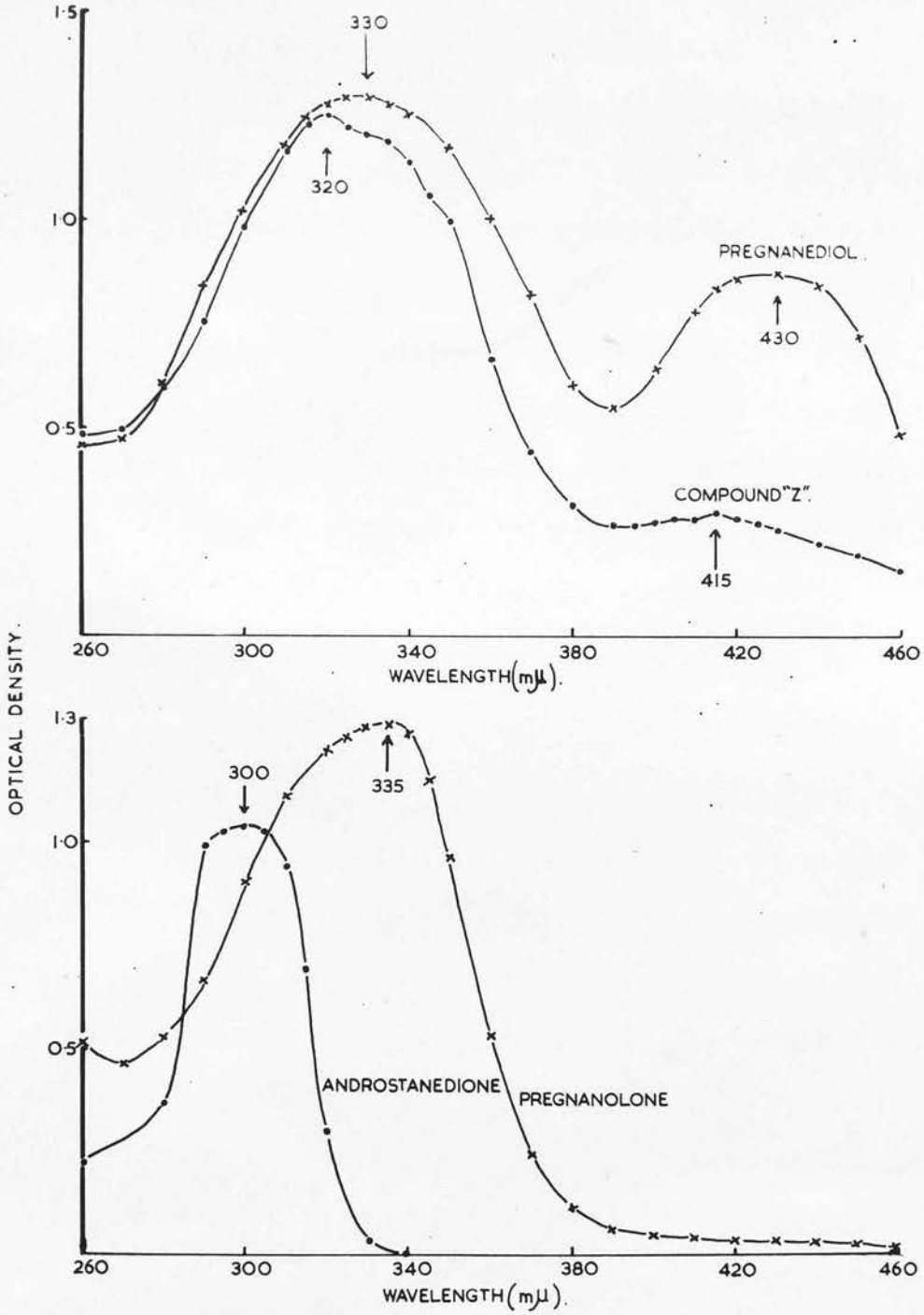


FIG.10. ABSORPTION SPECTRA OF SULPHURIC ACID-TREATED STEROIDS.

The three crystalline specimens were combined and recrystallised once more from aqueous acetone. The resultant crystals (2.6 mg., m.p. 124 - 130°) were dissolved in 25 ml. ethanol and measured volumes of the solution were subjected to a series of tests:-

i) 265 µg. yielded no significant amount of formaldehyde on oxidation with periodate.

ii) 265 µg. gave a negative Zimmermann reaction.

iii) At a concentration of 88 µg./ml. in ethanol, no significant absorption in the ultraviolet was obtained.

iv) 530 µg. gave no precipitate with digitonin.

v) With concentrated H_2SO_4 a faint yellow colour was produced at a concentration of 64 µg. "Z"/ml. conc. H_2SO_4. The absorption spectra of "Z" (64 µg./ml.) and of pregnanediol and pregnanolone (64 µg./ml.) and of androstane-3:20-dione (32 µg./ml.) are shown in Fig. 10. (Androsterone was also investigated but, to avoid congestion of the figures, the spectrum is not recorded here. The spectrum was of the same general type as that of androstanedione with absorption maximum at 305 mµ. except that there was some absorption over the range, 400 - 460 mµ.).

The shape of the spectrum for "Z" suggests that mainly pregnane derivatives are present. The significant absorption in the region 400 - 440 mμ. might indicate the presence of a small amount of "pregnanediol". However, no definite conclusions can be drawn concerning the identity of "Z" from the result of this and the other tests.

The mother-liquors from the many crystallisations were combined, evaporated to dryness and dissolved in a minimum volume of 90% ethanol. Treatment of this solution with digitonin resulted in the formation of a slight but definite precipitate. The α- and β- fractions so obtained are under investigation.

SECTION III

GENERAL DISCUSSION

i) Introduction

It has been shown that rabbit and rat liver contain an enzyme system capable of destroying progesterone. That this destruction is enzymic in nature is indicated by the finding that the activity of homogenate was destroyed by heating for 10 min. at 100° (p. 86) and was influenced by pH (p. 90). The rapidity of progesterone disappearance (approximately 50% in 30 min.) indicates that metabolism is not due to bacterial action (p. 93). The failure to obtain increased recovery of progesterone from incubation experiments when acid hydrolysis was incorporated into the extraction procedure shows that the disappearance of the steroid is not a result of the formation of an acid-labile conjugate (p. 91). Szego (1953) has shown that liver can catalyse the conjugation of oestrone or its metabolites with serum protein in vitro. This protein-bound oestrogen was released in ether-soluble form only after partial hydrolysis of the protein. Since in the present case, acid hydrolysis was not applied until after protein precipitation, the possibility was not eliminated that the progesterone was being "bound" by the liver protein to form a complex from which the steroid could not be removed by washing

with acetone. That such "binding" was not wholly responsible for the disappearance of progesterone on incubation of that steroid with liver is shown by the isolation of metabolites (p. 106).

In agreement with the findings of Wiswell et al. (1953), citrate has been found to cause increased progesterone metabolism (p. 96). Wiswell et al. have attributed this effect to the formation of complexes between citrate and other tricarboxylic acids and inhibiting metallic ions, since other metal-complex forming substances such as cyanide and cysteine also are activators of the progesterone metabolising system. It is the author's intention to investigate this point further by the use of other chelating agents such as "versene" (ethylene-diamine tetra-acetate), cf. Altmann and Crook (1953).

The failure of other members of the tricarboxylic acid cycle alone (p. 97) and in the presence of ATP and of ATP (p. 98) to cause any increase in progesterone metabolism indicates that "high-energy" phosphate bonds are not directly involved.

ii) The effect of DPN and nicotinamide

The ability of homogenate to metabolise progesterone was greatly increased by the addition of DPN and nicotinamide. This activating effect was more marked in rat liver (p. 83) than in rabbit liver (p. 79). In order to demonstrate that the activation was due to the added DPN and to protection of indigenous DPN by the added nicotinamide and not to the nicotinamide alone, the comprehensive experiment described on p. 85 was carried out. The results of this experiment are summarised in Fig. 8, the legend for which is given below.

Legend for Figure

Progesterone recovered

A. From "controls"

B. After incubation without additions.

C. Pre-incubation for 30 min. without additions.

D. 40 mM-nicotinamide (added before pre-incubation).

E. 40 mM-nicotinamide (added after pre-incubation)

F. 3.0 mM-DPN (added before pre-incubation).

G. 3.0 mM-DPN (added after pre-incubation).

H. 40 mM-nicotinamide and 3.0 mM-DPN; no pre-incubation.

(Double lines at end of bars indicate duplicate determinations)

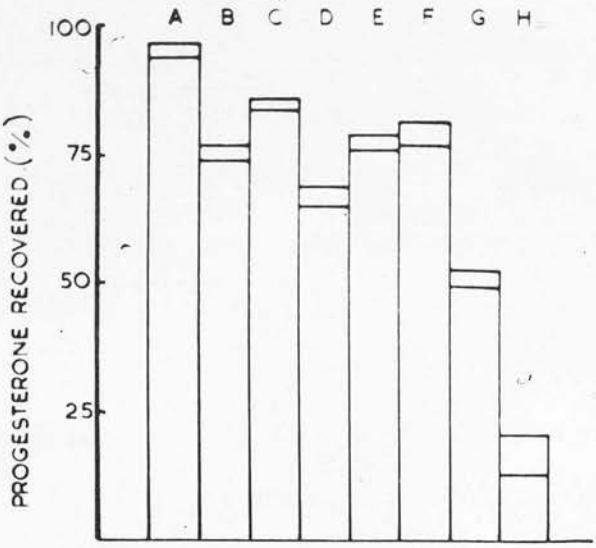


FIG.8. INCUBATION OF PROGESTERONE WITH RAT-LIVER HOMOGENATE (for details see text).

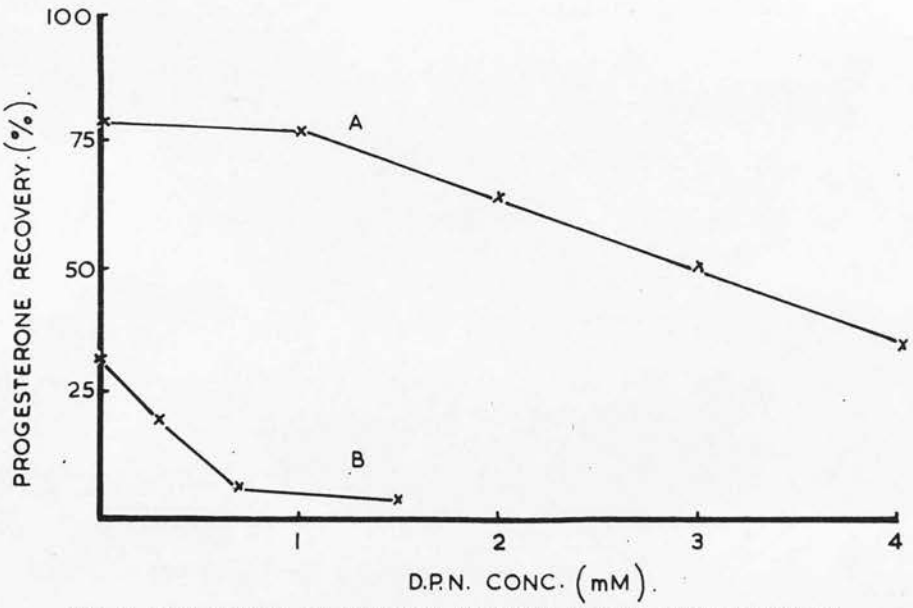


FIG.9. INFLUENCE OF D.P.N. ON METABOLISM OF PROGESTERONE BY RAT-LIVER HOMOGENATE.

A. WITHOUT ADDED NICOTINAMIDE.

B. PLUS 40 mM-NICOTINAMIDE.

It may be observed that there is a decrease in metabolism when homogenate is pre-incubated (bar C) compared with metabolism in homogenate not pre-incubated (bar B). This decrease is overcome by adding nicotinamide before (bar D) or DPN after (bar G) pre-incubation. It is not prevented by adding DPN before pre-incubation (bar F). The failure of nicotinamide added after pre-incubation to affect the degree of metabolism (bar E) shows that nicotinamide itself is not responsible for increased progesterone metabolism, but that it acts by protecting indigenous DPN from destruction by nucleosidase. The observation that pre-incubation without added DPN or nicotinamide (bar C) did not completely inactivate the homogenate suggests that alternative pathways exist for the metabolism of progesterone and that one or more is not DPN-sensitive.

The effect of varying concentrations of added DPN and nicotinamide is shown in Fig. 9. It appears that relatively high concentrations of DPN alone are required to obtain a degree of metabolism in homogenate comparable to that obtained with the same weight of tissue as slices (p. 81). The DPN used was only 37 per cent pure (see p. 10) and it is possible that it contained impurity capable of

inhibiting the enzymes involved in progesterone metabolism. Adenylic acid is the chief impurity in the DPN as prepared (Le Page, 1949) but when this substance replaced DPN in the reaction mixture, it had no effect on the activity of the homogenate (p. 97). Other adenine derivatives, however, might well be present in the DPN and these substances have been shown to be inhibitors of enzymes requiring DPN (Williams, 1953). It can also be seen from Fig. 9 that at a nicotinamide concentration of 40 mM in the final reaction mixture, indigenous DPN is almost completely protected.

In view of this strong evidence that DPN is involved in the metabolism of progesterone by rat liver, it is difficult to understand the failure of Wiswell et al. (1953) to observe any marked increase in metabolism in rat liver homogenates containing 40 mM-nicotinamide and 0.006 mM-DPN.

iii) The significance of the formaldehydogenic material formed after incubation of progesterone with liver

Some indication has been obtained that progesterone may be converted into formaldehydogenic substance (FSS) when incubated with liver (p. 99).

It is felt by the author that these results should be viewed with some reserve. Values obtained from "blank" and "control" experiments were very high even when progesterone was added in ethanol instead of propylene glycol, itself formaldehydogenic. In some cases, individual values from incubation experiments were lower than individual "blank" and "control" values, though incubation values were in general higher than the mean of "blanks" and "controls". Duplicate values were often widely different and no correlation appeared to exist between the amount of progesterone metabolised and the amount of FSS formed. It is important to note, however, that only in incubation experiments was the true "formaldehyde colour" obtained in the final colour reaction with chromotropic acid (results marked *). In the "blanks" and "controls", the final colour was always brown.

It is reasonable to assume, therefore, that FSS is produced on incubating progesterone with liver, though it does not necessarily follow that the FSS is steroidal or is produced directly from progesterone. The FSS might arise from "side reactions" not involving steroid and which might occur in the presence of progesterone but not in its absence.

The violet spots obtained after paper chromatography of incubated rabbit liver extracts on heating with 15 per cent phosphoric acid appear to indicate the formation of two steroids which are more polar than progesterone and are of the "corticosteroid" type. Further studies with rabbit liver and a more reliable paper chromatographic method than the "chromatocoil" are required to confirm these findings.

iv) The nature of the metabolic products

Two metabolic products, 5α-pregnane 3:20-dione (30 mg.) and 5α-pregnan-3α-ol-20-one (20 mg.) have been isolated and fully characterised (p. 106). Unchanged progesterone from the incubation experiments amounted to 12 mg. and the as yet unidentified semi-crystalline substance "Z" weighed 22 mg. Thus, only about 28% of the added progesterone (300 mg.) has been accounted for. While some of the loss is no doubt manipulative (only about 56% of added progesterone was recovered from the "control"), it is apparent that it reflects enzymic degradation of progesterone to substances not recoverable by the isolation technique employed. Substances more

"polar" than DOC might be present in the as yet un-investigated fractions in tubes 5 - 8 of the counter-current distribution (p. 111). That such substances may have been formed has been indicated by the finding that formaldehydogenic substance is produced when progesterone is incubated with liver (p. 99).

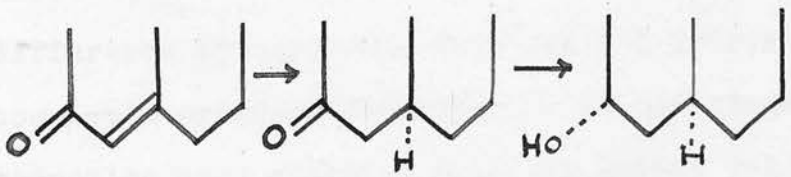
No 'pregnanediols' have been detected with certainty in the large scale experiment. It is not known whether the rat excretes 'pregnanediols' normally or after progesterone administration but the possibility of isolating 'pregnanediol' is reduced when it is considered that pregnanediol itself is metabolised by rat liver homogenate (Grant, 1952). The failure of 'pregnanediols' to accumulate, assuming that they are formed to any extent *in vitro*, would indicate that the rate of 'pregnanediol' metabolism must be equal to or greater than the rate of its formation. It is one of the limitations of *in vitro* experiments with homogenates, that metabolites may undergo further change during incubation whereas *in vivo*, the products of metabolism may be rapidly excreted from the cell and hence removed from the influence of enzymes able to metabolise them further.

Thus, the isolation of 5α-pregnanedione and 5α-pregnanolone does not provide proof that these substances are likely to be the main metabolites excreted by the rat *in vivo*. Furthermore, it is possible that *in vivo*, conjugation of the metabolites with glucuronic or sulphuric acid also occurs. Conjugation of the C-3 hydroxyl group *in vivo* might hinder re-oxidation at this position and also, might influence the reduction of the C-20 ketonic group in some way. Conjugation of steroid alcohols by liver has not been demonstrated *in vitro* and probably played no part in the incubation experiments of the present investigation.

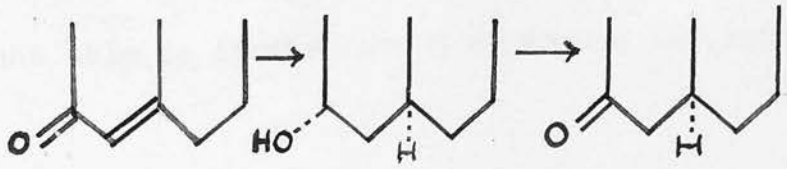
It is of interest that the reduction of the double bond in Ring A appears to be sterically directed to yield only 5α-pregnane derivatives. This finding is in agreement with that of Schneider (1952) who isolated 5α-pregnane derivatives but no pregnane (5β) derivatives after incubation of DOC with rat liver and with that of Caspi, Levy and Hechter (1953) who demonstrated that only 5α-pregnane compounds are formed when cortisone is reduced by perfusion through rat liver. In the present work, however, only 5α-pregnan-3α-ol-20-one was isolated whereas Caspi *et al.* and Schneider found that both the 3α- and 3β-isomers were formed. Indeed, in Schneider's

work, the main reduction product of DOC was found to be 5α-pregnane-3β:21-diol-20-one and only a small amount of the 3α-compound was formed. It would seem that the presence of an oxygen function at C-21 directs the steric course of the reduction of the C-3 ketonic group to the 3β-position. Schneider also isolated as a minor metabolite, 5α-pregnane-3:20-dione-21-ol whereas in the present work the corresponding dione from progesterone was a major metabolite. Schneider has put forward the suggestion, without experimental evidence to support it, that the dione obtained from DOC arises as a result of enzymic re-oxidation of the C-3 hydroxyl compound. Now the reduction of the A Ring of progesterone to yield 5α-pregnanedione and 5α-pregnanolone could occur:-

i) By reduction of the α-β-unsaturated ketonic group in two stages:



or ii) By one-stage reduction of the carbonyl group and the double bond followed by re-oxidation of the hydroxyl group:



It is tempting to postulate that reaction ii) is the more likely to occur. DPN could be assigned the role of "hydrogen" acceptor in the second stage, the re-oxidation of 5α-pregnanolone to 5α-pregnanedione. This stage may require oxygen since Grant et al. (1950) and Grant (1952) found that destruction of pregnanediol by rat liver was increased by addition of DPN and did not occur under anaerobic conditions. It should be possible to test this postulate by ascertaining whether or not a large amount of 5α-pregnanedione is formed when progesterone is incubated under anaerobic conditions and without added DPN or nicotinamide. Furthermore, whereas progesterone was incubated with homogenate and yielded 5α-pregnanedione as a major metabolite, Schneider incubated DOC with slices and obtained the corresponding dione as a minor metabolite. It is possible to account for this difference by supposing that the C-3 hydroxylated compounds produced from the DOC by one-stage reduction were excreted from the intact cells of the slices and hence were not available for re-oxidation.

It is of interest that Grant (1952) was not able to isolate any C-3 ketonic products after

he incubated pregnanediol with rat liver homogenate under aerobic conditions and with added DPN and nicotinamide. This may be accounted for by supposing that the enzyme responsible for oxidation of the fully reduced A Ring is specific for the 5-α configuration. The metabolism of 5α-pregnanediol by rat liver homogenates and slices would therefore repay investigation.

v) The reduction of non-benzenoid steroid hormones

It is clear then that rat liver contains an enzyme system capable of bringing about the reductive metabolism of progesterone. Testosterone (Samuels et al., 1947), methyl testosterone (Levedahl and Samuels, 1950) and DOC (Schneider, 1952) also undergo a similar change on incubation with rat liver. These steroids all possess an α-β unsaturated ketonic group in Ring A.

Table VIII summarises the conditions which influence the reductive metabolism of the steroids listed, by rat liver in vitro. It can be seen that different enzyme systems appear to be involved for each steroid. Thus, the enzyme system reducing Ring A of progesterone does not require oxygen while

Table VIII. Conditions affecting metabolism of Ring A of steroids by rat liver

Steroid	Activators	Effect of absence of O_2	Products
Testosterone(a)	Citrate DPN	No metabolism	(see text)
Methyl testosterone (b)	Citrate	No metabolism	Not investigated
DOC (c)	(DPN-see text)	Slight inhibition	5α-pregnanes 3β-ol mainly
Progesterone (d)	Citrate DPN	No effect	5α-pregnanes 3α-ol mainly

(a) Samuels et al. (1947)
(b) Levedahl and Samuels (1950)
(c) Schneider et al. (1951)
Schneider (1952)
(d) Present investigation

that reducing this ring in the other steroids does. Citrate markedly influences the metabolism of all the steroids listed except DOC but the mechanism of the action of this activator does not appear to be the same in the case of testosterone and progesterone (Wiswell et al., 1953). The enzymes responsible for the reduction of testosterone appear to be less active than those reducing DOC and progesterone since Clark and Kochakian (1947) isolated mainly androst-4-ene-3:17-dione and 17β-testosterone after incubation of testosterone with rat liver. Only a very small amount of C-3 hydroxylated material was detected. In the case of progesterone and DOC on the other hand, reduction of Ring A is rapid and appears to be the major reaction involved. Finally, the steric course of the reduction of the carbonyl group appears to differ in progesterone and DOC since the main C-3 hydroxylated from the former is 3α and from the latter, 3β.

The author considers, however, that these differences are more apparent than real, especially in the case of progesterone and DOC. Schneider et al. (1951) obtained 19.4 per cent reduction of the A Ring of DOC in the presence of 1.0 mM-DPN compared with 14.4 per cent reduction by homogenate alone,

at a tissue:steroid ratio of 250:1. This difference they regard as not significant. In the author's work with progesterone, 1.0 mM-DPN (37 per cent pure) had no effect, alone, on the activity of homogenates but when nicotinamide (40 mM) was also present the activity of homogenate was greatly increased. Schneider et al. do not record the purity of the DPN used. That DPN may indeed be involved in DOC metabolism is also indicated by Schneider's finding that homogenate was less active than slices (11.2 and 34 per cent metabolism respectively). Fish, Hayano and Pincus (1953) have reported that addition of DPN increases the reduction of the A Ring of cortisone by rat liver. Thus, further investigation may eventually show that DPN plays a general role in the metabolism of the C_{21} steroid hormones.

Enzyme purification studies and investigations on the localisation of the "A Ring enzyme" by means of differential centrifugation of tissue homogenates, would provide results of great interest.

vi) Some general observations on the metabolism of progesterone

It is well known that certain quantitative

and qualitative differences exist among species as regards excretion of pregnanediol and related compounds (Pincus and Pearlman, 1943). Of particular interest in relation to the present work is the finding of Marker and Rohrmann (1939) that whereas no 'pregnanediols' were isolated from the urine of pregnant sows, 'pregnanolones' were isolated. It is not known whether the rat excretes 'pregnanediols' normally or after progesterone administration. The author's finding that little or no 'pregnanediol' is formed after incubation of progesterone with rat liver suggests that the enzyme responsible for the reduction of the C-20 carbonyl group may be lacking in this organ of the rat. Thus, it is possible that *in vivo*, the rat metabolises progesterone only as far as 'pregnanolones'. It is a matter of some importance then, that the metabolism of progesterone by the rat *in vivo* should be investigated. Also, large scale isolation experiments *in vitro*, employing the livers of animals known to excrete pregnanediol (rabbit, cow and human) should be carried out. Such experiments would provide information regarding the steric course of the reduction of the A Ring of progesterone and of the activity of the C-20 ketone reducing system in the livers of such species.

The role of other organs, especially the "target" organs in the metabolism of progesterone requires full investigation. The rapid destruction of the hormone by rat liver indicates that this organ can play a major part in the inactivation of the steroid but it does not follow that the liver is the major site of metabolism *in vivo*. The exploratory experiments described in Section I of this Thesis indicated that pregnanediol is not produced on incubation of progesterone with rabbit uterus. The method used for the determination of pregnanediol was rather insensitive and it would be unwise to assert on the basis of these experiments, that pregnanediol is not formed under the conditions employed. No information was obtained from these experiments as to whether the progesterone had been converted to products other than pregnanediol. The metabolism of progesterone by other tissues therefore requires investigation, with particular reference to the uteri of ovariectomised and oestrogen-treated, ovariectomised animals.

In view of the finding that 5α-pregnanedione and 5α-pregnanolone are the main products obtained after incubation of progesterone with rat liver, the

determination of these steroids and their pregnane analogues in urines is obviously a major need. The use of pregnanediol determinations as an index of progesterone metabolism must be viewed with some reserve if less hydrogenated derivatives of the hormone are also major metabolites. Indeed, Pearlman and Cerceo (1953) have reported the presence in human pregnancy urine (6 months) of 46 mg. of 20-ketosteroids of the "5α-pregnanolone type" in a 24 hr. sample. At the same stage of pregnancy, about 50 mg. pregnanediol are excreted per day (Venning, 1938). The method used by Venning gave high values for pregnanediol since the "purified" sodium pregnanediol" glucuronidate which was weighed in the final analysis has been shown to be contaminated with about 20 per cent of sodium pregnanolone glucuronidate (Sutherland et al., 1947). Thus, it is possible that if all of the metabolic reduction products of progesterone could be determined in urine, a high proportion of administered hormone could be accounted for. It is possible, of course, that the urine may not be the main pathway for the excretion of progesterone metabolites and so urinary studies will need to be supplemented by examination of bile and faeces.

In vivo studies on progesterone metabolism are complicated by the possible relationship of this steroid with the adrenal cortical hormones (see p. 5) and also by the production from adrenal cortical hormones, of steroids such as pregnanediol and related compounds (Heard, 1948).

vii) Conclusion

The problem of progesterone metabolism thus provides biochemists with a subject of great interest and importance and one which will not be readily solved. Studies on excreta and on blood, especially investigations with isotopically labelled steroid; perfusion experiments and other *in vitro* techniques will all play their part in providing information. Until we have gained some insight into the biochemical mechanisms involved in progesterone metabolism we cannot hope to understand much about the causes of the variations which occur in the metabolism of this steroid in different species and in health and disease.

APPENDICES

APPENDIX I

Partition coefficients

As a preliminary to the development of a solvent system suitable for the separation of progesterone from liver extracts (p. 59) and for counter-current distribution (p. 111), the partition coefficients of the steroid in a number of solvent systems were determined.

The solvent systems were equilibrated at 17° overnight and 5 ml. volumes of upper and lower phase pipetted into a glass-stoppered test tube containing 0.5 mg. steroid. After standing for at least 2 hr. with periodic shaking, 2 ml. portions of upper and lower phase were removed for analysis. Determinations were carried out in duplicate.

Partition coeffients of progesterone at 17 ±0.5

$$K = \frac{\text{Concn. in aqueous methanol}}{\text{Concn. in "organic" phase}}$$

A. Aqueous methanol: n-hexane

Methanol (%)	K
60	1.2, 1.0
65	1.6, 1.6
70	2.0, 1.9
75	2.4, 2.8

B. Aqueous methanol: n-hexane, benzene

Methanol (%)	Benzene (%)	K
70	5	1.6, 1.4
70	10	1.0, 0.86
80	80	0.24, 0.26
85	80	0.54, 0.50

APPENDIX II

Metabolism of progesterone by rat liver mitochondria

A study of the influence of steroids on the *in vitro* synthesis of citrate from pyruvate and malate by rat liver mitochondria has been undertaken by Dr. J.K. Grant in this department. Progesterone was found to be a potent inhibitor of citrate synthesis (Grant and Taylor, 1952). It was therefore of interest to ascertain whether or not progesterone itself was being metabolised while acting as an inhibitor of citrate synthesis. Since the present author was concerned only with the steroid analyses, no attempt will be made to discuss the broader aspects of this subject.

Methods

The citrate-synthesising system of Kennedy and Lehninger (1949) was employed, the constituents of the final reaction mixture being as follows:- 0.05 M-KCl, 0.005 M-$MgSO_4$, 0.01 M-phosphate buffer (pH 7.4), 0.001 M-cytochrome c, 0.0016 M-K pyruvate, 0.0016 M-K malate, 0.0005 M-ATP; mitochondria suspension containing about 1 mg. nitrogen. Final volume, 3.0 ml. The progesterone was added in 0.01 - 0.02 ml. propylene glycol. For the controls,

progesterone was added to the incubated reaction mixture and the flasks immediately processed. Incubation was in air for 2 hr. at 37°.

Progesterone was determined in the manner described for homogenates except that it was not found necessary to chill the flasks during precipitation of protein by acetone.

Results

a) Incubations with added pyruvate and malate

Expt. No.	Progesterone added (µg.)	Progesterone recovered (corr. for blank) After incubn. (µg.)	(%)	From controls (µg.)	(%)
1.	215	195	91	205	95
		195	91	207	96
2.	197	177	90	195	99
		175	89	190	98
3.	177	171	97	172	97
		172	97	171	97
4.	165	125	76	139	84
		125	76	150	90
	330	217	66	297	90
		237	72	300	91
5.	194	162	84	179	92
		164	85	190	99
6.	86	81	94	83	97
		79	93	85	99

b) Incubations with and without pyruvate and malate

Experiment No.	Progesterone recovered (corr. for blank) 1.		2.	
	(µg.)	(%)	(µg.)	(%)
Progesterone added	182		125	
After incubation				
With pyruvate and malate	130	71	110	88
	165	91	102	82
No pyruvate and malate	160	88	119	95
	175	96	117	94
From controls	175	96	120	96
	180	99	116	93

Discussion

It is apparent that incubation of progesterone with rat liver mitochondria results in a slight destruction of the steroid. The results obtained show great variation, however, and there does not appear to be any correlation between the degree of metabolism and the amount of steroid added. In one experiment (No. 3) no progesterone metabolism took place yet in this and the other experiments, citrate synthesis was completely or almost completely inhibited. The two experiments in which progesterone was incubated with and without pyruvate

and malate appear to indicate that destruction of progesterone depends on the presence of these acids.

In view of these experiments and of the findings reported in Section II, it would be of interest to determine the localisation of the enzymes metabolising progesterone, in the liver cell. Such an investigation has been carried out on the oestrogen-inactivating system of rat liver by Riegel and Meyer (1952). Rat liver was separated into nuclear, mitochondrial, microsomal and "supernatant" fractions by differential centrifugation. The original homogenate, the fractions and combinations were tested for their ability to metabolise oestrogens. No single fraction was very active while the microsomes and "supernatant" when combined, were comparable in activity to the original homogenate.

APPENDIX III

Purification of organic solvents

Most of the methods are those described by Weissberger and Proskauer (1935). The fractionating columns used in redistillations were packed with Raschig rings and were either 40 x 3.5 cm. ("long column") or 20 x 2 cm. ("short column"). Final distillations were carried out in all-glass apparatus and solvents were collected under anhydrous conditions.

Acetone: Commercial acetone was refluxed with potassium permanganate (10 g./l.) and NaOH (4 g./l.) for 2 hr. After distillation, the acetone was refluxed for 2 hr. with freshly baked potassium carbonate (50 g./l.) and then distilled through the long column and stored in brown bottles.

Benzene: A.R. grade benzene was dried with sodium wire and fractionally distilled through the short column.

Chloroform: B.P. grade chloroform was washed 4 x 0.25 vol. water, dried over sodium sulphate, A.R., distilled and stored in brown bottles away from sunlight.

Diethyl ether: The A.R. grade was shaken with ferrous

sulphate in H_2SO_4 (600 g. $FeSO_4.7H_2O$, 60 ml. concentrated H_2SO_4 in 1100 ml. water), washed with N-NaOH solution and then with water until the washings were neutral. After drying over anhydrous calcium chloride, the ether was distilled and stored in the dark.

Ethanol: Absolute ethanol was refluxed with NaOH pellets (5 g./l.) for 8 hr., distilled, fractionated through the long column, and stored in the dark.

Ethanol, aldehyde free: Ethanol treated as described above was allowed to stand for at least a week in the dark over m-phenylenediamine hydrochloride (2 g./l.) with periodic shaking. The ethanol was distilled as required through a short column.

Ethyl acetate: The A.R. grade was washed with 1/20 vols. N-HCl N-sodium bicarbonate (1/20 vol.) and water until the washings were neutral, dried over anhydrous sodium sulphate, A.R., and redistilled.

n-Hexane: "Aromatic free" grade hexane was allowed to stand over concentrated H_2SO_4 A.R. (50 ml./l.) for 12 hr. with occasional shaking. After washing with N-NaOH solution and water until the washings were neutral, the hexane was left overnight in contact with anhydrous calcium chloride and then filtered. After further drying with sodium wire, the hexane was distilled through the short column.

Cyclohexane (Hexahydro-benzene): Commercial cyclohexane was washed with water, dried over anhydrous calcium chloride and fractionally distilled through the short column.

Methanol: Absolute methanol was refluxed with NaOH pellets (10 g./l.) for 12 hr., distilled and redistilled through the long column and stored in dark bottles.

APPENDIX IV

Numerical details of Figures

Table 1. Analysis of fractions from partition columns (Fig. 2)

Eluate (ml.)	Progesterone (µg.)								Column
	A	B	C	D	E	F	G	H	
11	-	-	-	-	-	-	-	-	
12	-	-	-	-	-	-	-	-	
13	-	-	-	-	-	-	-	-	
14	2	-	-	-	2	-	-	-	
15	7	2	2	2	5	3	2	3	
16	24	12	10	12	15	18	10	5	
17	28	18	20	25	22	26	30	31	
18	6	8	17	10	12	15	10	20	
19	-	6	4	3	3	-	-	2	
20	-	-	-	-	-	-	-	-	
21	-	-	-	-	-	-	-	-	
22	-	-	-	-	-	-	-	-	

Table 2. Absorption spectra of "progesterone fractions" from incubation and "blank" experiments (Fig. 4)

Wavelength	Optical Density x 10^3	
(mμ.)	"Blank"	Incubation
230	51	438
235	40	500
239	40	525
240	40	530
241	40	525
245	39	485
250	40	410
255	39	300
260	39	180
265	35	110
270	22	60
275	20	45
280	15	41
285	13	40
290	10	35
295	10	34
300	10	30

Table 3. Counter-current distribution of extracts from isolation experiment

Fig. 5 Incubations

Tube No.	Weight (mg.)		Weight Fractions	
	(1)	(2)	(1)	(2)
0	31.3	19.2	0.12	0.08
1	32.9	46.0	0.13	0.20
2	25.9	31.4	0.10	0.13
3	19.6	19.2	0.07	0.08
4	11.4	13.2	0.04	0.05
5	8.9	8.0	0.03	0.03
6	11.2	8.1	0.04	0.03
7	20.5	11.2	0.08	0.04
8	87.2	66.4	0.35	0.29

(1) and (2) refer to incubation experiments 1 and 2: see p. 107.

Fig. 6

	Control		Blank	
	Weight mg.	Weight Fraction	Weight mg.	Weight Fraction
0	50.1	0.17	14.0	0.03
1	56.5	0.19	12.1	0.02
2	42.3	0.14	7.8	0.01
3	21.5	0.07	7.0	0.01
4	10.8	0.03	5.8	0.01
5	7.0	0.02	23.0	0.04
6	9.9	0.03	41.0	0.09
7	24.4	0.08	90.7	0.21
8	76.0	0.25	234.0	0.53

Table 4. Adsorption chromatograms of tubes 0 - 4 of counter-current distributions (Fig. 7)

Fraction No.	Solvent.	Wt. in mg. Incubation	Incubation	Control	Blank
1	Ben:Hexane	11.0	43	15	47
2	"	-	-	-	-
3	"	-	-	-	-
4	"	-	-	-	-
5	"	-	-	-	-
6	"	-	-	-	-
7	Benzene.	2.6	-	-	-
8	"	16.4	2.0	0.7	0.8
9	"	6.9	4.5	0.3	1.6
10	"	3.7	4.1	-	-
11	"	3.8	1.8	-	-
12	"	2.0	1.6	-	-
13	"	2.4	1.7	-	-
14	"	1.4	1.2	-	-
15	"	1.9	1.1	-	-
16	"	0.8	1.2	23.4	0.8
17	Ether:Benzene	1.2	1.3	45.8	0.3
18	"	2.7	1.7	22.3	0.2
19	"	2.8	-	4.2	-
20	"	1.0	-	2.1	-
21	Ethanol:Ben.	42.7	40.0	1.4	0.6
22	"	-	-	-	-
23	"	-	-	-	-
24	"	-	-	-	-
25	Ethanol	-	-	-	-

Table 5. Absorption Spectra of steroids treated with concentrated H_2SO_4. (Fig. 10)

Wavelength (mμ.)	Optical density			
	Pregnane-diol	Pregnano-lone	Androstane-3:20-dione	"Z"
260	0.46	0.50	0.22	0.48
270	0.52	0.46	0.36	0.49
280	-	0.52	-	0.60
290	0.84	0.66	0.90	0.75
295	0.93	0.78	1.03	0.86
300	1.01	0.90	1.04	0.98
305	-	-	1.02	-
310	1.17	1.11	0.94	1.16
315	1.24	1.21	0.69	1.23
320	1.27	1.22	0.30	1.25
321	-	-	-	1.28
325	1.29	1.25	-	1.22
330	1.29	1.28	0.03	1.20
335	1.27	1.28	-	1.18
340	1.25	1.26	-	1.14
345	-	1.15	0.0	1.05
350	1.17	0.96	0.0	0.94
360	1.00	0.53	0.0	0.66
370	0.82	0.24	0.0	0.44
380	0.60	0.11	0.0	0.31
390	0.55	0.06	0.0	0.26
400	0.64	0.05	0.0	0.27
410	0.78	0.04	0.0	0.28
415	0.84	-	0.0	0.29
420	0.86	0.03	0.0	0.28
430	0.87	0.03	0.0	0.25
440	0.84	0.03	0.0	0.22
450	0.72	0.02	0.0	0.20
460	0.48	0.01	0.0	0.16

APPENDIX V

REFERENCES

Altman, S.M. and Crook, E.M. (1953). Nature, London 171, 76.

Archibald, R.M. (1946). J.biol.Chem. 165, 443.

Axelrod, L.R. and Miller, L.L. (1953). Fed.Proc. 12, 173.

Bischoff, F. and Katherman, R.E. (1948). Amer.J. Physiol. 152, 189.

Bischoff, F. and Pilhorn, H.R. (1948). J.biol.Chem. 174, 663.

Bock, L.H. and Rainey, J.L. (1948). U.S. Patent 2454541.

Bradbury, J.T., Brown, W.E. and Gray, L.A. (1950). Rec. Prog. in Horm. Res. 5, 151.

Brendler, H. (1951). Science 114, 61.

Brownie, A.C., Grant, J.K. and Taylor, W. (1953). Biochem.J. (in press).

Bush, I.E. (1952). Biochem.J. 50, 370.

Butt, W.R., Morris, P., Morris, C.J.O.R. and Williams, D.C. (1951). Biochem.J. 49, 434.

Callow, N.H., Callow, R.K. and Emmens, C.W. (1938). Biochem.J. 32, 1312.

Cantarow, A., Paschis, K.E., Williams, T.L. and Havens, W.P. (1952). Fed. Proc. 11, 22.

Caspi, E.Y., Levy, H. and Mechter, O.M. (1953). Arch. Biochem. Biophys. 45, 169.

Clark, L.C. and Kochakian, C.D. (1947). J.biol.Chem. 170, 23.

Cohen, P.P. (1945). In Umbreit, W.W., Burris, R.H. and Stauffer, F.J. Manometric Techniques and Related Methods for the Study of Tissue Metabolism. Minneapolis: Burger Publishing Co.

Coppedge, R.L., Segaloff, A. and Sarrett, H.P. (1950). J.biol.Chem. 182, 181.

Craig, L.C. and Craig, D. (1950). Technique of Organic Chemistry, Vol. 3. Interscience Pub. New York.

Deutsch, W. (1936). J.Physiol. 87, 56P.

Diczfalusy, E., Ekwall, P., Sjoblom, L. and Westman, A. (1952). Act. endocrinol. 9, 387.

Diczfalusy, E. and Westman, A. (1950). Act. endocrinol. 5, 275.

Dobriner, K., Lieberman, S. and Rhoads, C.P. (1948). J.biol.Chem. 172, 241.

Dorfman, R.I., Ross, E. and Shipley, R.A. (1948). Endocrinology 42, 77.

Ekwall, P., Lundsten, T. and Sjoblom, L. (1951). Act.chem.Scand. 5, 1383.

Ekwall, P. and Sjoblom, L. (1949). Acta chem. Scand. 3, 1174.

Ekwall, P. and Sjoblom, L. (1950). Act. endocrinol. 4, 179.

Engel, P. (1944). Endocrinology 35, 70.

Ercoli, A. and Koller, M. (1949). 1st International Congress Biochemistry, Cambridge. p. 391.

Fieser, L.F. (1929). J.Amer.chem.Soc. 46, 2639.

Fish, C.A., Hayano, M. and Pincus, G. (1953). Arch. Biochem. Biophys. 42, 480.

Friedgood, H.B. and Garst, J.B. (1948). Recent Prog. Horm. Res. 2, 31.

Friend, D. and Hastings, A.B. (1940). Proc.Soc.exp. Biol.Med. 95, 137.

Glassman, H.N. (1950). Science 111, 688.

Grady, H.J., Elliot, W.H., Doisy, E.A. Jr., Bocklage, B.C. and Doisy, E.A. (1952). J.biol.Chem. 195, 755.

Grant, J.K. (1950). Thesis presented for the Degree of Doctor of Philosophy, University of Edinburgh.

Grant, J.K. (1952). Biochem.J. 51, 358.

Grant, J.K. and Marrian, G.F. (1950). Biochem.J. 47, 497.

Grant, J.K. and Taylor, W. (1952). Biochem.J. 52, xxiv.

Handler, P. and Klein, J.R. (1942). J.biol.Chem. 143, 49.

Haskins, A.L. (1950). Proc. Soc. exp. Biol. N.Y. 73, 439.

Hayano, M. and Dorfman, R.I. (1952a). Fed.Proc.11, 228.
(1952b). Arch. Biochem. 36, 237.
(1953). J.biol.Chem. 201, 175.

Heard, R.D.H. (1948) in the Hormones. Pincus, G. and Thimann, Vol.1, p.619. Academic Press, New York.

Hechter, O., Zaffaroni, A., Jacobsen, R.P., Levy, H., Jeanloz, R.W., Schenker, V. and Pincus, G. (1951). Rec. Prog. in Horm. Res. 6, 215.

Hoffman, M.M. and Browne, J.S.L. (1942). Canad. Med. Ass. J. 47, 424.

Jones, R.N. (1948). Rec.Prog.Horm.Res. 2, 3.

Jones, A.S., Webb, M. and Smith, F. (1949). J.chem. Soc. p. 2164.

Kahnt, F.W. and Wettstein, A. (1951). Helv.chem.Act. 34, 1790.

Kennedy, E.P. and Lehninger, A.L. (1949). J.biol. Chem. 179, 957.

Klyne, W. and Patterson, J.Y.F. (1948). Biochem.J. 42, i.

Klyne, W. and Rankeillor, J. (1947). Chem. & Ind. 17, 224.

Kochakian, C.D. (1951). Ann. New York Acad. Sci. 54, 534.

Kochakian, C.D. and Aposhian, H.V. (1952). Arch. Biochem. 37, 442.

Krebs, H.A. (1950). Biochim. biophys. Acta 4, 249.

Krebs, H.A. and Eggleton, L.V. (1940). Biochem.J. 34, 442.

Kritchevsky, D. and Kirk, M.R. (1952). Arch. Biochem. 35, 346.

Kyle, T.I. and Marrian, G.F. (1951). Biochem.J. 49, 162.

Lambden, M.P. (1950). Fed. Proc. 9, 193.

Le Page, G.A. (1947). J.biol.Chem. 168, 623.

(1949). Biochemical Preparations, I, 28. New York. John Wiley.

Levedahl, B.H. and Samuels, L.T. (1951). J.biol. Chem. 186, 857.

Lieberman, S. (1946). Experimentia 2, 411.

Lieberman, S. and Dobriner, K. (1948). Rec.Prog. in Horm. Res. 3, 71.

Lieberman, S., Dobriner, K., Hill, B.R., Fieser, L.F. and Rhoads, C.P. (1948). J.biol.Chem. 172, 263.

MacLeod, J. and Rhoads, C.P. (1940). Proc.Soc.exp. Biol.Med. 41, 268.

Mann, J.P.C. and Quastel, J.H. (1941). Biochem.J. 35, 502.

Marker, R.E., Kamm, O. and McGrew, R.V. (1937). J.Amer.chem.Soc. 59, 616.

Marker, R.E. and Rohrmann, E. (1939). J.Amer.chem. Soc. 61, 3476.

Marrian, G.F. (1949). Rec. Prog. in Horm. Res. 4, 1.

Marrian, G.F. and Gough, N. (1946). Biochem.J. 40, 376.

Neher, R. and Wettstein, A. (1951). Helv.chem.Acta. 34, 2278.

Patterson, J.Y.F. and Marrian, G.F. (1953). Acta Endocrinol. 13, 1.

Pearlman, W.H. (1948). in the Hormones, Vol. I, p. 407. Academic Press. New York.

Pearlman, W.H. and Cerceo, E. (1948). J.biol.Chem. 176, 847.

Pearlman, W.H. and Cerceo, E. (1953). J.biol.Chem. 203, 127.

Pearlman, W.H. and De Meio, R.H. (1949). J.biol. Chem. 179, 114.

Pearlman, W.H. and Pincus, G. (1946). Fed. Proc. 5, 79.

Pincus, G. and Pearlman, W.H. (1943). Vitamins & Hormones I, 293.

Plager, J.E.and Samuels, L.T. (1952). Fed. Proc. 11, 383.

(1953). Arch. Biochem. 42, 477.

Potter, V.R. and Elvehjem, C.A. (1936). J.biol. Chem. 114, 495.

Putnam, F.W. (1948). Advanc. Protein Chem. 4, 79.

Riegel, L and Meyer, R.K. (1952). Proc.Soc.exp.Biol. Med. 80, 617.

Riegel, B., Hartop, W.L. Jr., and Kittinger, G.W. (1950). Endocrinology 47, 311.

Rogers, J. and McLellan, F. (1951). J. clin. Endocrinol. 11, 246.

Samuels, L.T. (1947). J.biol.Chem. 168, 471.

Samuels, L.T., McCaulay, C. and Sellers, D.M. (1947). J.biol.Chem. 168, 477.

Samuels, L.T. and West, D. (1952). Vitamins & Hormones, 10, 251.

Savard, K. (1953). J.biol.Chem. 202, 457.

Schneider, J.J. (1952). J.biol.Chem. 199, 235.

Schneider, J.J. and Horstman, P.M. (1951). J.biol. Chem. 191, 327.

Schneider, J.J. and Horstman, P.M. (1952). ibid 196, 629.

Schneider, J.J. and Mason, H.L. (1948,a). J.biol. Chem. 172, 771

Schneider, J.J. and Mason, H.L. (1948,b). ibid 175, 231.

Schwarz, V. (1952). Biochem.J. 52, xiii.

Seneca, H., Ellenbogen, E., Henderson, E., Collins, A and Rochenbach, J. (1950). Science. 112, 524.

Sommerville, I.F., Gough, N. and Marrian, G.F. (1948). J.Endocrinol. 5, 247.

Spaulding, M.E. and Graham, W.D. (1947). J.biol. Chem. 170, 711.

Stern, R. and Bird, L.H. (1949). Biochem.J. 44, 635.

Sutherland, E.S. and Marrian, G.F. (1947). Biochem. J. 41, 193.

Sweat, M.L. and Samuels, L.T. (1948). J.biol.Chem. 173, 433.

Szego, C. (1953). Endocrinology. 52, 669.

Umbreit, W.W., Burris, R.H. and Stauffer, J.F. (1949). Manometric Techniques for the Study of Tissue Metabolism. 2nd ed. Burgess Pub. Co. Minneapolis.

Venning, E.H. (1938). J.biol.Chem. 126, 597.

Walker, P. (1951). Biochem.J. 49, 620.

Warren, C.O. (1944). J.biol.Chem. 156, 559.

(1947). ibid 167, 543.

Weissberger, A. and Proskauer, E. (1935). Organic Solvents, Oxford; Clarendon Press.

Williams, J.N. (1953). J.biol.Chem. 195, 629.

Williams, R.T. (1949). Detoxication Mechanisms; Chapman & Hall, London.

Wiswell, J.G. and Samuels, L.T. (1953). J.biol. Chem. 201, 155.

Zimmermann, W. (1935). Z. physiol. Chem. 233, 257.

Zondek, B. (1941). Investigations on the Genital Functions and their Regulations. Williams & Wilkins Co. Baltimore.

APPENDIX VI

Publications

[*Reprinted from the Proceedings of the Biochemical Society*, 19–20 *September* 1952.
Biochem. J., 1952, Vol. 52, Part 4, p. xxiv.]

The Influence of Steroids on Citrate Metabolism. By J. K. Grant and W. Taylor. (*Biochemistry Department, University of Edinburgh*)

Components of the tricarboxylic acid cycle have been implicated in the *in vitro* metabolism of steroid hormones. Thus Hayano & Dorfman (1952) reported that fumarate, malate or succinate accelerated C-11 hydroxylation of corticosteroids by adrenal homogenates. Sweat & Samuels (1948) observed that citrate increased the disappearance of the $\alpha\beta$ unsaturated ketonic grouping of testosterone on incubation with liver. The intervention of steroid hormones in the operation of the tricarboxylic acid cycle was suggested by observations of Du Bois, Cochran & Zerwick (1951) that citrate accumulates in livers of fluoroacetate-poisoned female rats but not in females receiving testosterone nor in males.

The influence of various steroids on the *in vitro* metabolism of citrate has now been studied.

Using mitochondria from liver of ovariectomized rats, and with pyruvate and malate as substrates (Kennedy & Lehninger, 1949), testosterone and androsterone (600 μM) caused 60 % (9) and 50 % (2) inhibition of citrate synthesis. The following C_{21} steroids tested at concentrations of 130 and 260 μM caused inhibition of citrate synthesis: progesterone 79 % (15), 86 % (7); 17-hydroxy-progesterone, 1·5 % (3), 16 % (2); 17-hydroxy-deoxycorticosterone, 0 % (5), 33 % (6); deoxycorticosterone, 39 % (6), 76 % (3); cortisone and corticosterone (130 μM) appear to increase citrate synthesis 24 % (4) and 39 % (6). CoI in concentrations up to 0·01 M had no effect on the inhibition obtained with 130 μM progesterone. (Figures in parentheses are the numbers of experiments.) Citrate was determined by an adaptation of the method of Weil-Malherbe & Bone (1949). Similar results were obtained with oxaloacetate as substrate. In a typical experiment the oxygen uptake of the system was reduced 88 % by 130 μM progesterone. In four experiments 9 to 21 % of added progesterone (measured by absorption at 240 mμ. following partition chromatography) disappeared while citrate synthesis was inhibited 80 %. In a fifth experiment no change in progesterone was observed although citrate synthesis was strongly inhibited. These results confirm in part and extend recent observations of Cochran & Du Bois (1952) on the inhibitory action of steroid hormones on citrate synthesis. The mechanism of inhibition is under investigation.

REFERENCES

Cochran, K. W. & Du Bois, K. P. (1952). *Fed. Proc.* **11**, 333.

Du Bois, K. P., Cochran, K. W. & Zerwick, M. M. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 452.

Hayano, M. & Dorfman, R. I. (1952). *Fed. Proc.* **11**, 228.

Kennedy, E. P. & Lehninger, A. L. (1949). *J. biol. Chem.* **179**, 957.

Sweat, M. L. & Samuels, L. T. (1948). *J. biol. Chem.* **173**, 433.

Weil-Malherbe, H. & Bone, A. D. (1949). *Biochem. J.* **45**, 377.

Enzymic 11-β-Hydroxylation of 11-Deoxycorticosterone (DOC). By A. C. Brownie, J. K. Grant and W. Taylor. (*Biochemistry Department, University of Edinburgh*)

Various workers (Hayano & Dorfman, 1953; Kahnt & Wettstein, 1951; Sweat, 1951 have studied the 11-β-hydroxylation of steroids by adrenal preparations, and have demonstrated a requirement for fumarate or certain other members of the tricarboxylic acid cycle (Krebs's cycle) as 'activators' of hydroxylation. Satisfactory evidence for the mechanism of this activation is lacking.

The 11-β-hydroxylation of DOC has now been studied, using washed mitochondria prepared in sucrose from fresh beef adrenal cortical tissue, incubated aerobically in phosphate buffer (pH 7·4) with Mg^{2+}. DOC was determined by absorption at 240 mμ, following recovery from incubation mixtures in $95 \pm 3\%$ yield, by solvent partition procedures. No DOC metabolism occurred in the absence of Krebs's cycle acids. Pyruvate was without effect. Other Krebs's cycle acids (0·002 M) activated hydroxylation. 0·0005 M-ATP enhanced this effect, but was without effect itself. In presence of ATP, α-ketoglutarate, succinate and fumarate were most effective, hydroxylating 0·9–1·0 μmole DOC/mg. mitochondria total N in 1 hr. at 37°. l-Malate, oxalacetate and citrate were less effective. The product appeared to be almost entirely corticosterone as judged by total steroid recovery and comparison with authentic corticosterone run on paper chromatograms with various solvent systems.

Fumarate failed to activate DOC hydroxylation under anaerobic conditions. Aerobically, increasing succinate concentrations increased DOC hydroxylation, supporting the view that the reaction fumarate → succinate is not involved, and that fumarate does not function as a hydrogen acceptor.

Observations of O_2 consumption indicated that DOC hydroxylation is related to the rate of oxidation of Krebs's cycle acids present, but these studies are complicated by inhibition of respiration by the DOC.

2:4-Dinitrophenol, widely employed as an uncoupler of phosphorylation and oxidation (Loomis, & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949), used in concentrations which did not inhibit oxidation of Krebs's cycle acids, caused marked inhibition of DOC hydroxylation.

It thus appears highly probable that the role of Krebs's cycle acids is to support oxidative phosphorylation for the 'sparking' of the steroid to be hydroxylated (cf. Lehninger, 1945*a*, *b*). Fumarate is not specifically involved in this role, nor does it appear to be an essential hydrogen acceptor.

REFERENCES

Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.

Hayano, M. & Dorfman, R. I. (1953). *J. biol. Chem.* **201**, 175.

Kahnt, F. W. & Wettstein, A. (1951). *Helv. chim. acta*, **34**, 1790.

Lehninger, A. L. (1945*a*). *J. biol. Chem.* **157**, 363.

Lehninger, A. L. (1945*b*). *J. biol. Chem.* **161**, 437.

Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.

Sweat, M. L. (1951). *J. Amer. chem. Soc.* **73**, 4056.